이학박사학위논문

CD4/CD8 T세포 계열 결정 과정에서 Twist2의 기능 연구

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2017년 2월

서울대학교 대학원

생명과학부

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Studies on the function of Twist2

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ABSTRACT

Studies on the function of Twist2 during CD4/CD8 thymocyte lineage commitment

Sunsook Hwang

CD4/CD8 T cell lineage commitment is one of the central issues in the development of the immune system. T cell receptor (TCR) signal strength and/or duration is known to be a key in determining the lineages. Several transcription factors are known to participate during CD4/CD8 lineage commitment. Gata3 and ThPOK reportedly play critical roles in CD4 single-positive (CD4 SP) differentiation. Conversely, Runx3 and Mazr reportedly play important roles in CD8 single-positive (CD8 SP) differentiation. Particularly, ThPOK seems to be both necessary and sufficient for CD4-lineage differentiation and Runx3 plays an important role in CD8 SP differentiation by repressing ThPOK expression. However, the critical factor(s) regulating this process is still missing. For example, a defined factor(s) translating the TCR signal and co-receptor complexes into the lineage differentiation is not clear yet. Also, it has been reported that the Runx complex binds persistently to the silencer region of *ThPOK* in thymocyte subsets, regardless of their capability to differentiate into the CD4 or CD8 SP lineage. Thus, it has been postulated that some other factor(s) would act to

resolve the distinct suppressive activity of the Runx complex on *ThPOK* between CD4 and CD8 SP cells.

Twist2 is a basic helix-loop-helix transcription factor that generally act as transcriptional repressors. It was reported that Twist2 expression was found to be regulated by TCR signaling and to adjust the survival or death of thymocytes and Twist2 controls TCR-mediated apoptosis by regulating the expression of Nur77 and Nor-1. These results suggest that Twist2 may play an important role during lymphocyte development.

Here, I show that Twist2 is a critical factor for CD4/CD8 thymocyte differentiation. Twist2 expression is differentially regulated by T-cell receptor signaling leading to differentiation into the CD4 or CD8 lineage. Twist2 expression was higher in CD8 SP thymocytes than in CD4 SP thymocytes. Twist2 interacts with Runx3 and their interaction is dependent on Twist2 carboxyl-terminal Twist-box. Also, Twist2 downregulated the expression of ThPOK like Runx3. Twist2 could bind to the silencer region of the ThPOK promoter in a lineage-dependent manner. ThPOK silencer has two important regions, which are an essential 80bp core sequence (containing NFkb and E box) and a Runx binding domain. Twist2 was specifically bound to the Runx sites. These results suggest that Twist2 interacts with Runx3 to bind to the RBS1 site of *ThPOK*, thereby blocking its expression. Moreover, Twist2 transgenic mice perturbed $CD4^+$ thymocyte differentiation, whereas enhanced $CD8^+$ thymocyte differentiation. In contrast, the development of CD8 SP thymocytes was impaired in Twist2 deficient mice. Also, Twist2 expression

produced mature $CD8^+$ thymocytes in $B_2m^{-/-}$ mice, while its deficiency significantly impaired $CD8^+$ cells in TCR transgenic mice favoring CD8 T-cell differentiation. These gain-of-function and loss-of-function mutations mice systems showed that Twist2 has a critical role in CD4/CD8 thymocyte lineage differentiation through interaction with Runx complex to suppress the expression of *ThPOK* in a CD8 SP lineage-specific manner.

Keywords:

Twist2, CD4/CD8 lineage commitment, ThPOK, Runx complex

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INTRODUCTION

I. Thymocyte differentiation

Bone marrow-derived progenitors for T cells expressing T cell receptors (TCRs) develop in the thymus through stages that are characterized by the expression of CD4 and CD8 co-receptors(Rothenberg and Taghon, 2005; von Boehmer, 2004). The earliest progenitors do not express either CD4 or CD8 (double negative; DN). DN thymocytes can be further subdivided into four stages based on the surface expression of CD44 and CD25 molecules: $CD44^{+}CD25^{-}$(DN1), $CD44^{+}CD25^{+}$(DN2), $CD44^{-}CD25^{+}$(DN3), and $CD44^{-}CD25^{-}$(DN4)(Ceredig and Rolink, 2002). TCR β-chain gene rearrangement occurs during DN3 stage. After successful TCR β-chain gene rearrangement, DN3 thymocytes further differentiate into the DN4 ($CD44^{-}CD25^{-}$) stage, followed by the intermediate single-positive (ISP) and $CD4^{+}CD8^{+}$ double-positive (DP) stages. DP thymocytes go through positive and negative selection depending on the interaction of their TCRs with the major histocompatibility complex (MHC) expressed on thymic stromal cells(Doyle and Strominger, 1987; Norment et al., 1988). Only a small fraction of DP thymocytes can differentiate into CD4 single-positive (CD4 SP) or CD8 single-positive (CD8 SP) cells.

The lineage decision of DP thymocytes between CD4 SP and CD8 SP has been investigated intensively(Bosselut, 2004; Kappes et al., 2005; Laky and Fowlkes, 2005; Seong et al., 1992; Singer and Bosselut, 2004). Although several models have been proposed, it is generally well accepted that the match between MHC classes and the TCR with a CD4 or CD8 co-receptor is critical for differentiation. MHC class II-selected thymocytes differentiate into CD4 SP committed to the helper lineage, whereas thymocytes expressing MHC class I–restricted TCRs differentiate into CD8 SP thymocytes committed to the cytotoxic lineage. TCR signaling from the CD4 co-receptor was shown to be stronger than that from the CD8 co-receptor, and Lck tyrosine kinase was suggested to play an important role during this signal transduction(Hernandez-Hoyos et al., 2000; Lovatt et al., 2006). The high kinase activity of Lck redirects MHC class I-restricted thymocytes into the CD4 SP thymocyte lineage, whereas low kinase activity generates MHC class II-restricted CD8 SP thymocytes. These results suggest that kinase activity near proximal TCR signaling is critical for CD4/CD8 lineage commitment. In addition, the kinetic modulation of CD4 and CD8 co-receptors has been proposed to be important for differentiation(Singer, 2002). Thus, it appears that signal transduction from TCR is critical for CD4/CD8 lineage differentiation.

II. Transcription factor in lineage commitment

Some transcription factors may link proximal TCR signaling to the CD4/CD8 lineage commitment process(He et al., 2005; Hernandez-Hoyos et al., 2003; Sun et al., 2005a; Wilkinson et al., 2002; Woolf et al., 2003). Tox is essential for the transition from positively selected thymocytes to the $CD4^{+}CD8^{lo}$ stage where lineage commitment may be determined and maintaining or upregulating CD4 expression in positively selected DP thymocyte(Aliahmad and Kaye, 2008; Aliahmad et al., 2004). In TOX deficient mice, positively selected thymocytes do not differentiate into $CD4^{+}CD8^{lo}$ cells but instead become $CD4^{lo}CD8^{lo}$ cells that fail to develop into CD4 SP cells, whereas CD8 SP T cell development is almost normal (Aliahmad and Kaye, 2008). Gata3 and ThPOK (T-helper-inducing POZ/Kruppel- like factor; also known as Zbtb7b or cKrox; called "ThPOK" hereafter) reportedly play critical roles in CD4 SP differentiation. GATA3 is induced by TCR signaling in DP cells(Hernandez-Hoyos et al., 2003) and preferentially expressed by $CD4^{+}$ T cells(Hendriks et al., 1999). In addition, Gata3 overexpression blocks the generation of CD8 SP cells (Nawijn et al., 2001; Pai et al., 2003), whereas Gata3 deficiency severely decreased CD4 SP cell numbers without affecting CD8 SP generation(Pai et al., 2003). However, GATA3 overexpression does not redirect MHC class I-restricted thymocytes into the CD4 SP cells, which indicated that GATA3 is not a CD4 lineage specifying factor(Hernandez-Hoyos et al., 2003). ThPOK deficiency or a mutation in the *ThPOK* gene results in the complete perturbation of CD4 SP development and redirection of MHC class II-signalled thymocytes to the CD8 SP cell, whereas ThPOK overexpression in the thymus redirects MHC class I-

restricted thymocytes to the CD4 SP thymocyte lineage(Dave et al., 1998; He et al., 2005; Sun et al., 2005a). Conversely, Runx3 and Mazr reportedly play important roles in CD8 SP differentiation. Runx3 can suppress both CD4 co-receptor and ThPOK expression by directly binding to the silencer region of both genes(Setoguchi et al., 2008; Taniuchi et al., 2002). Runx1 and Runx3 double deficient mice impair CD8 SP development, whereas T cell specific Runx3 transgenic mice increase the proportion of CD8 SP thymocytes(Grueter et al., 2005; Kohu et al., 2005; Woolf et al., 2003). Also, Mazr represses ThPOK expression in MHC class I–signaled DP thymocytes via binding to the ThPOK silencer and thus prevents redirection of CD8 SP cells into the CD4 lineage(Sakaguchi et al., 2010) (**Figure 1**).

III. ThPOK

The transcription factor ThPOK is a zinc-finger protein that is encoded by the *Zbtb7b* (also known as *Zfp67*) gene. Two laboratories first discovered that ThPOK is important for CD4 lineage differentiation(He et al., 2005; Sun et al., 2005a). One of them used cDNA microarray tool to compare mRNA expression in intrathymically TCR signaled thymocytes ($CD69^+$ DP cells) and $CD69^-$ unsignaled DP thymocytes(Sun et al., 2005a). Excluding a number of molecules that had been previously published, one of the most highly upregulated genes was *Zfp67*. They also found ThPOK expression was greater in CD4 SP than in CD8 SP thymocytes and CD8 SP cell development was

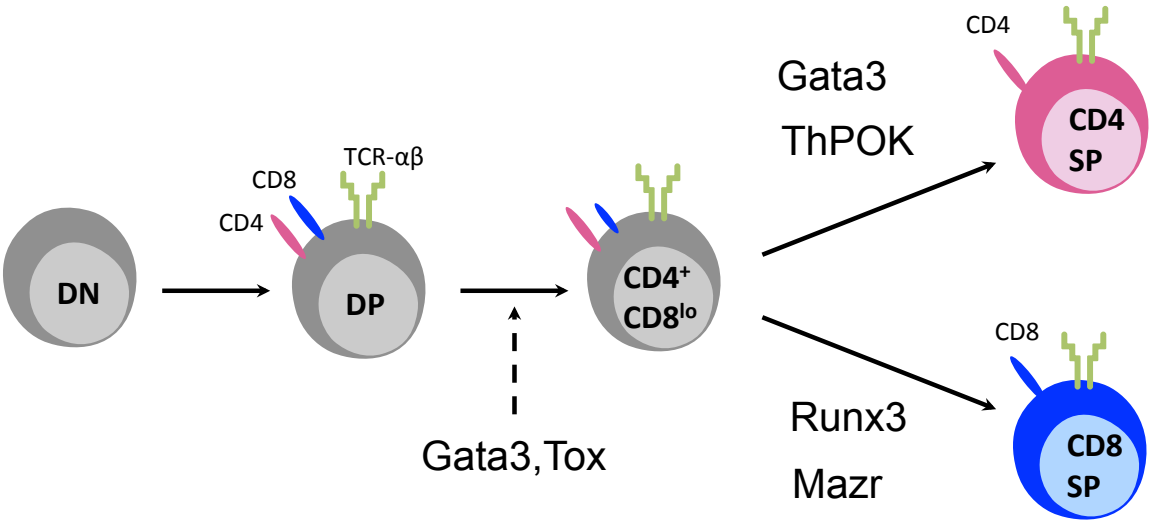


Figure 1. Overview of TCR signal cues and transcription factors that influence CD4/CD8 lineage differentiation.

impaired and MHC class I-restricted thymocytes differentiated into $CD4^+$ T cells with helper properties in ThPOK transgenic mice. Another team identified a spontaneous recessive mutation in mice, the HD (helper deficient) mutation. This mutation caused redirection of MHC class-II-signaled thymocytes to the CD8 SP lineage whereas no detectable TCR signalling defects in HD mice(Keefe et al., 1999). They identified a point mutation which A to G change at 1165 position within the ThPOK coding region, resulting in an Arg to Gly change at amino acid position 389(He et al., 2005). This mutation caused mutation in the second zinc finger domain of ThPOK and presumably disrupts DNA binding. According to results of two laboratories, ThPOK seems to be both necessary and sufficient for CD4-lineage differentiation(Kappes et al., 2006).

ThPOK is essential to maintain CD4 T cell integrity in peripheral $CD4^+$ T cells. Cytotoxic effector gene, perforin, Granzyme B and Eomes, elevated in ThPOK reduced peripheral $CD4^+$ T cells. However, enforced expression of ThPOK in peripheral $CD8^+$ T cells induces $CD4^+$ T cell lineage features(Jenkinson et al., 2007). Also, it was reported that ThPOK maintains *Cd4* gene transcription by antagonizing silencer activity through binding to CD4 silnencer region(Muroi et al., 2008), whereas it decreases *Cd8* gene expression by downregulating the enhancer activity of E8I(Jenkinson et al., 2007).

IV. Regulatory region of ThPOK gene locus

Two laboratories identified potential cis-acting elements within the *ThPOK* fragment(He et al., 2008; Setoguchi et al., 2008). He et al. (2008) performed DNase I Hypersensitive Site (DHS) analysis and transgenic reporter assays. They found six DHS sites. Among them, distal regulatory element (DRE) acts as a repressor and general T lymphoid element (GTE) and proximal regulatory element (PRE) act positive regulatory elements of ThPOK. Especially, DRE is important site for suppression of ThPOK expression in MHC class I-restricted thymocytes, because the deletion of DRE resulted in reporter expression in both the CD4 and CD8 lineages. In addition, deletion and mutagenesis analysis defined an essential 80bp core sequence of DRE. The 80 bp core region is essential for silencer function and contains both an NFkB and E-box motif. They suggest the model that ThPOK transcription is repressed by a DRE-dependent mechanism at the DP stage and CD8 SP stage, and MHC class II-TCR signaling converts the DRE from silencer to enhancer mode and initiates ThPOK transcription at the $CD4^{+}8^{lo}$ stage. In CD4 SP cells, ThPOK expression is maintained by a TCR independent mechanism and maybe required the GTE and PRE elements(He et al., 2008).

Setoguchi et al. (2008) identified two major cis-regulatory elements of ThPOK using transgenic reporter assay and ChIP-on-chip experiments. To understand mechanisms of Runx-mediated ThPOK repression, they performed ChIP-on-chip experiment whether Runx complexes directly bind to the ThPOK locus, and then identified two binding regions of Runx complexes in the

ThPOK locus. Distal Runx-binding sequences (RBS1) and proximal Runx-binding sequences (RBS2) are located ~3.1 kb upstream and ~7.4 kb downstream of exon Ia. To understand the function of RBS1 and RBS2, they performed transgenic reporter assays, identifying RBS1 (also called ThPOK silencer) acts as a transcriptional silencer and RBS2 (also called Proximal enhancer) acts as an enhancer. RBS1 domain overlaps DRE region. To identify the physiological function of the RBS1, they produced RBS1- or RBS2-deleted mice. Deletion of RBS1 from one ThPOK allele resulted in the loss of peripheral $CD8^+$ T cells and derepression of ThPOK in $CD69^-$ DP thymocytes. Thus, they concluded that Runx-dependent silencer activity inhibits ThPOK expression and $CD4^+$ T cell development, resulting in cytotoxic T cell differentiation. Deletion of the RBS2 lead to inefficient upregulation of ThPOK, resulting in the transdifferentiation of helper lineage cells into the cytotoxic T cell lineage. However, Runx complexes always bind to RBS-1 and RBS-2 in both ThPOK–expressing and nonexpressing cells, so Runx binding to these regions did not correlate with ThPOK repression. Therefore, they suggest that additional molecules that interact with Runx complex bound to the ThPOK silencer may play a central role in ThPOK silencer activity(Setoguchi et al., 2008).

In addition, GATA3 induce ThPOK expression via binding to at least two sites within the ThPOK locus (Site A and SiteB)(Wang et al., 2008). Site A domain overlaps RBS2 region. Also, Recent studies reported that transcription factor MAZR binds to the ThPOK silencer region and regulates CD4/CD8

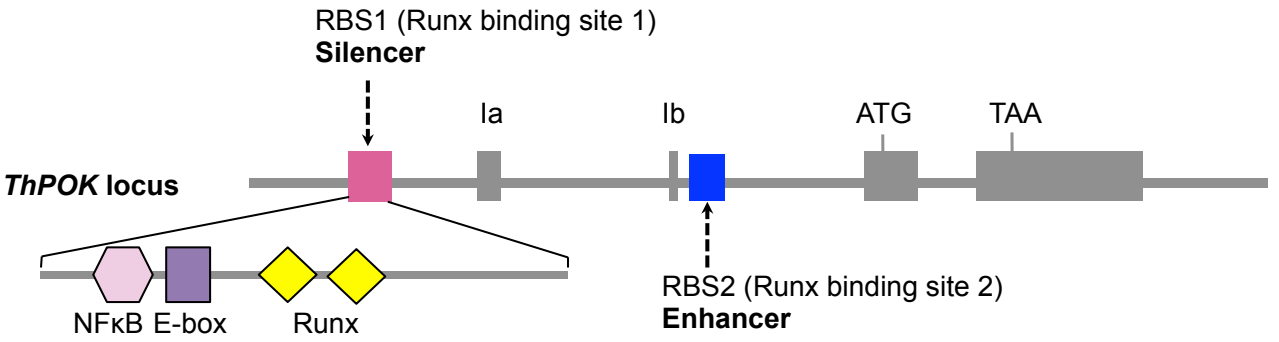


Figure 2. Regulatory regions of *ThPOK* gene locus.

lineage differentiation(Sakaguchi et al., 2010). (Figure 2 shows a schematic diagram of regulatory regions for ThPOK locus.)

V. Runx complex

Runx proteins contain a conserved 128 amino acid Runt DNA binding domain. Three mammalian Runt domain transcription factors have been identified, that is Runx1, Runx2, and Runx3(Levanon and Groner, 2004). Runx transcription factors are heterodimers from one of the three Runx proteins with a non–DNA-binding partner, Cbfb protein(Ito, 1999). Also, Runx proteins contain conserved Val-Trp-Arg-Pro-Tyr (VWRPY) motif that recruit the Groucho/TLE co-repressor proteins to their target genes(Aronson et al., 1997; Levanon et al., 1998). In the thymus, Runx1 is mainly expressed in DN thymocytes and is required to repress CD4 expression(Taniuchi et al., 2002). Runx3 is not expressed in pre-selected DP cells and its expression is induced by IL-7 cytokine signals during the differentiation of $CD4^{+}CD8^{lo}$ thymocytes into CD8 SP cells(Liu et al., 2005; Park et al., 2010). Runx1 and Runx3 can bind to the *Cd4* silencer region to suppress the *Cd4* gene expression(Taniuchi et al., 2002; Woolf et al., 2003) and bind to the E8I *Cd8* enhancer element to reinitiates *Cd8* expression(Sato et al., 2005). Also, recently, Runx complex was found to bind to silencer and enhancer regions of ThPOK(Setoguchi et al., 2008). Thus, Runx complex promotes CD8 SP differentiation through silencing of *Cd4* and *ThPOK* gene and reinitiation of *Cd8*.

VI. Mazr

Myc-associated ZF-related factor (MAZR; also known as Patz1 or Zfp278 and encoded by the Patz1 gene) is member of the family of zinc-finger factors that contain a BTB (POZ) domain(Bilic and Ellmeier, 2007). MAZR binds to several *Cd8* cis-regulatory elements and acts as one of the factors involved in keeping the *Cd8* loci in a transcriptional off state in DN thymocytes(Bilic et al., 2006).

MAZR interacts with Runx proteins through C-terminal ZF7 domain. In preselected DP thymocytes, MAZR and Runx1 together repressed ThPOK, whereas in $CD8^+$ T cells, MAZR and Runx3 synergized suppression of ThPOK expression. MAZR is also required for the maintenance of ThPOK repression in peripheral $CD8^+$ T cells(Sakaguchi et al., 2010). Also, Runx1-MAZR and Runx3-MAZR double knock out mice showed that increased *Cd4* derepression in DN and in CD8 SP, respectively(Sakaguchi et al., 2015). Therefore, this is indicated that synergistic activities between Runx complexes and MAZR could repress the CD4 gene(Naito et al., 2011; Sakaguchi et al., 2015; Sakaguchi et al., 2010).

VII. Twist2

Twist2 is a class II basic helix-loop-helix transcription factor that was discovered in Drosophila as an E12 binding protein(Li et al., 1995). There are

two murine homologs of *Drosophila* Twist: Twist1 and Twist2(Sosic et al., 2003). Twist2 dimerizes with class I bHLH proteins like E proteins, then binds to E box domains. Murine Twist proteins generally act as transcriptional repressors. Also, Twist2 expression is related to the mesenchymal cell lineage such as osteoblast, myocytes, and adipocytes and Twist2 involved in early embryogenesis, bone, muscle, and adipocyte differentiation, and epithelial-mesenchymal transition(Li et al., 1995). For example, Twist2 inhibits the osteoblast differentiation in preosteoblast(Tamura and Noda, 1999) and represses the transcriptional activity of MEF-MyoD during myogenesis(Gong and Li, 2002). Also, Twist2 is highly expressed in fat tissue and inhibits transcriptional activity of ADD1/SREBP1c, so involves in adipocyte differentiation(Lee et al., 2003). These studies show that Twist2 plays an important role in lineage differentiation of various tissues.

Twist2-deficient mice show growth retardation and perinatal death due to cachexia(Sosic et al., 2003). Twist2 knock out mice show increased apoptotic bodies in the thymus and spleen and also increased cytokine production, proinflammatory gene expression and myeloid lineage differentiation(Sharabi et al., 2008; Sosic et al., 2003). Twist2 binds to cytokine promoters and inhibits their activity. Recently, Twist2 expression was found to be regulated by TCR signaling and to adjust the survival or death of thymocytes. Twist2 expression is induced via the Ca^{2+}–Cacineurin–NFATc3 pathway through moderate TCR signal of positive selection, whereas NFATc3-dependent Twist2 expression is abolished via specific activation of the JNK pathway through strong TCR signal of negative selection(Oh et al., 2016). Also Twist2 controls TCR-

mediated apoptosis by regulating the expression of Nur77 and Nor-1(Oh et al., 2016). These results suggest that Twist2 may play an important role during lymphocyte development. Here, I show that Twist2 is a critical player during DP thymocyte differentiation into mature CD4/CD8 SP T cells.

MATERIALS AND METHODS

Mice

Twist2 transgenic mice and Twist2 conditional knockout mice were raised in our laboratory and have been described(Oh et al., 2016). *ThPOK*-GFP mice(Setoguchi et al., 2008) were from Ichiro Taniuchi; *CD4* transgenic mice(Van Laethem et al., 2007) were from Alfred Singer; and *Runx3* transgenic mice(Kohu et al., 2005) were provided by Masanobu Satake. $Rag^{-/-}$ HY-TCR and $Rag^{-/-}$ DO transgenic mice were purchased from Taconic Farms (Korea). $Tcra^{-/-}$ mice and $B2m^{-/-}$ mice were obtained from the Jackson Laboratory. C57BL/6 mice were purchased from Charles River Laboratories. The mice were routinely screened by PCR with DNA obtained from tail tissue. Mice between 4-8 weeks old were used for experiments. The mice were bred and maintained under specific pathogen-free conditions, and experiments were performed in accordance with institutional and national guidelines.

Cell culture and transient transfection

293T cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE) supplemented with 10% FBS (WelGENE),

100U/ml streptomycin and penicillin. Twist2 and/or Runx3-expressing vector was transient transfected into 293T cells by Calcium phosphate transfection method following the manufacturer's instruction.

Bone marrow reconstitution assay

Bone marrow cells from *lck*-Cre *Twist2*$^{+/+}$ or *lck*-Cre *Twist2*$^{f/f}$ mice (CD45.2^{+}) were labeled with biotinylated anti-CD3ε (145-2C11; BD Bioscience), followed by negative depletion with anti-biotin bead (Miltenyi Biotec). T cell-depleted bone marrow cells were mixed at a ratio of 1:1 with T-cell depleted CD45.1^{+} bone marrow cells, and 8 $\times 10^{6}$ cells were injected in to the tail veins of lethally irradiated 8 week old *MHC class II*$^{-/-}$ mice (5.5Gy x 2; ^{137}Cs source, *NCIRF*). At 4 weeks after transplantation, mice were sacrificed and their cells were analyzed by flow cytometry (FACS Canto II).

Antibodies

PerCP-conjugated streptavidin, anti-CD3ε-PE, anti-CD4-PE, anti-CD4-PE-Cy7, anti-CD8-FITC, anti-CD8-APC-Cy7, anti-CD69-PerCP, anti-TCRβ-APC, anti-CD44-FITC, anti-CD25-APC, anti-CD24-PE-Cy7, anti-HY-TCR-APC, and anti-DO11.10-PerCPcy5.5 antibodies were purchased from BD Biosciences or eBioscience. Anti-Twist2 monoclonal antibody (H00117581-M01) was purchased from Abnova.

Flow Cytometry

Single cell suspensions were prepared from thymus, spleen, and lymph node and then passed through cell straniner to remove cell debris. The red blood cells were lysed by ACK lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, and 0.1mM EDTA). 3 x 10^6 cells were used for antibody staining. Stained cells were analyzed or collected on a FACSCanto II or a FACSAria II (BD Biosciences). For cell sorting, a single-cell suspension of thymocytes was stained using anti-CD4-PE, anti-CD8-APC-Cy7, anti-TCRβ-APC, anti-CD24-PE-Cy7 and anti-CD69-PerCP antibodies, and each subset of cells was sorted. The purity of all sorted populations was above 95%.

RT-PCR and Q-PCR

RNA was extracted from the sorted populations or tissues by Trizol reagent (Invitrogen) according to the manufacturer's instruction. RNA was reverse transcribed with SuperScript III (Invitrogen) or QuantiTect Reverse Transcription Kit (Qiagen). Semi-quantitative RT-PCR was conducted by using a PCR premix kit (ELPIS Biotech, Korea). Quantitative RT-PCR was conducted with Taqman Master mix (Applied Biosystems) and primers purchased from Assays-On-Demand (Applied Biosystems) for *Twist2*, *ThPOK,*

Runx3 and *GAPDH*. The results were quantified with StepOnePlus® (Applied Biosystems). *GAPDH* was used as an internal control for RT-PCR analysis.

Co-immunoprecipitation and western blotting

Myc-tagged Runx3 and/or HA-tagged Twist2 was transfected into 293T cells. Cells were lysed with IPLS lysis buffer (0.05 M Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, and 0.5% NP-40) supplemented with protease inhibitor cocktail (Roche). Cell lysates were immunoprecipitated with anti-Myc antibodies. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P membrane (Millipore). Blotted proteins were detected using antibodies to Twist2 (H00117581-M01, Abnova), Myc epitope (9E10, Roche), and HA epitope (HA-7, Sigma) with 5% skim milk in TBST (150mM NaCl, 10mM Tris-Cl, pH8.0, 0.5% Tween-20). Protein bands were visualized by chemiluminescence using Enhanced chemiluminescence (ECL) reaction kit (Elpis, Korea).

ChIP

Total 10^6 thymocytes were fixed with 1% formaldehyde for 10 min at 37°C. Cells were collected by centrifugation and washed twice in cold PBS containing protease inhibitors (1mM PMSF and protease inhibitor cocktail,

Roche) and lysed on ice for 10 min in 200µl of SDS lysis buffer (Upstate Biotechnology). The chromatin was sheared with an Ultrasonic processor XL (Misonix Inc.). The chromatin fragments are 400–600 base pairs in length. After adding 10 fold ChIP dilution buffer, to reduce nonspecific background, sonicated cell supernatants were precleared for 1h with salmon-sperm DNA/protein G Sepharose beads and then immunoprecipitated with anti-Twist2 (H00117581-M01, Abnova) or control mouse IgG1 purified antibodies (Invitrogen, MG100) overnight at 4°C. Immunoprecipitated complexes were collected on salmon-sperm DNA/protein G Sepharose beads for 1h, washed sequentially once with low salt buffer, high salt buffer, LiCl buffer and twice with TE buffer and then eluted with elution buffer. Elution sample incubated for 4h at 65°C with 5M NaCl and then for 1h at 45°C with proteinase K to reverse the crosslink. DNA was purified by QiAquick Spin Kit (Qiagen) and eluted DNA was analyzed by PCR with the following primers: RBS1, forward: 5'-CAG AAT AGG CGC GCA GTT A-3' and reverse: 5'-CTG GCT GGT CCA AGT ACA CA-3', RBS2, forward: 5'-CTA AAG AGC TGT GTG CTA GAC C-3' and reverse: 5'-GTT TCA GGC AGG TGA GGT TC-3', UP1, forward: 5'-TGT TTA GTT TGG CTT GAG CCC-3' and reverse: 5'-CCC TCC TAC TCG TTT CAA AC-3' and GAPDH intron, forward: 5’-TTA CTT TCG CGC CCT GAG-3’ and reverse: 5’-GCG GTT CAT TCA TTT CCT TC-3’.

Duolink *in situ* PLA analysis

Transfected HeLa cells or sorted thymocytes were used. The cells were fixed with 4% paraformaldehyde for 20 min, permiablized with 0.1% Triton X-100 for 5 min and then blocked with Duolink blocking buffer (Olink Bioscience) for 30 min at 37°C. The permeabilized HeLa cells/thymocytes were incubated with antibodies against Myc and HA/Twist2 (H00117581-M01; Abnova) and CBFβ (ab33516; Abcam) overnight at 4°C. For secondary antibodies, PLA probes conjugated with a unique short DNA strand (anti-rabbit PLUS and anti-mouse MINUS; Olink Bioscience) were added and incubated for 1h at 37°C. After ligation and circularization of the DNA, they were amplified via rolling circle amplification using polymerase for 1h at 37°C and the reactions were detected using a complementary Cy3-labeled DNA linker. The fluorescence signal was detected using confocal microscopy.

Biotinylated-DNA pull-down assay

Four oligonucleotides, containing biotin on the nucleotide at 5'-position, were used in the pull-down assays. The sequences of these oligonucleotides were as follows: Runx binding site (34mer) F: biotin-5'-GGG GCT GCG GTC TGA GCG CCC CCA GCG GTT TCC T-3'; NF-κB and E box (43mer) F: biotin-5'-GGA GGG GGT ACC CTT GGC AGC CAC CGC CTC TTC AGG TGG G TTG-3'; mut Runx binding site (34mer) F: biotin-5'-GGG GCG AAT

TCC TGA GCG CCC CCG AAT TCT TCC T-3'; mut NF-κB and E box (43mer) F: biotin-5'-GGA GGT TCT ACC CTT GGC AGC CAC CGC CTC TTT AGG TAG GTT G-3'. Each 2μg of double-stranded oligonucleotide was incubated with 100μg of nuclear proteins for 20 min at room temperature in a binding buffer containing 20% glycerol, 5mM $MgCl_2$, 50mM Tris (pH 7.5), 250mM NaCl, 2.5mM EDTA, and 10μg of poly(dI-dC) competitor. After incubation, 20ul of streptavidin-coated magnetic beads M-270 (Dynal®, Invitrogen) were added to the reaction and incubated at 4°C for 4h. The protein-DNA-streptavidin magnetic bead complex was washed three times with binding buffer and loaded onto a SDS gel. Bounding proteins in the pull-down material were analyzed by Western hybridization using antibodies recognizing HA and Myc-tag. Protein bands were visualized by chemiluminescence using Enhanced chemiluminescence (ECL) reaction kit (Elpis, Korea).

Intracellular staining

Thymocytes stained on CD4, CD8, TCRβ, CD24 and CD69 were fixed and permeabilized and further stained with IgG or anti- Twist2 antibodies using the Foxp3 Fix/Perm Kit (eBioscience) following the manufacturer's instruction. After sequential addition of the anti-mouse IgG-biotin and Streptoavidin-FITC antibody, intracellular stained Twist2 were detected on a FACSCanto II.

Statistical analysis

Statistical analysis was performed by Prism software (GraphPad). Two-tailed Student's *t-tests* were used to calculate p values. P values <0.05 were considered statistically significant.

RESULTS

Twist2 expression is induced by TCR signaling and is more marked in CD8 SP induced signal

To study the role of Twist2 in thymocyte differentiation, thymocytes at various developmental stages were sorted by flow cytometry, and *Twist2* expression was analyzed by quantitative real-time reverse transcription polymerase chain reaction (Q-PCR) analysis. After the DP stage, *Twist2* expression was increased at the $CD4^{+}CD8^{lo}$ ($CD4^{+}CD8^{lo}CD69^{hi}TCR\beta^{+}$; simplified "$CD4^{+}CD8^{lo}$" hereafter) stage, followed by distinct patterns of CD4 SP and CD8 SP thymocytes. *Twist2* expression in CD8 SP thymocytes was approximately 3 fold higher than in CD4 SP thymocytes (**Figure 3**). To investigate whether such differential expression of *Twist2* is reflected during CD4- or CD8-SP favored differentiation, thymocytes from $Tcra^{-/-}$ mice were activated with 0.2 ng/mL PMA and 0.2 μg/mL ionomycin (conditions leading to CD4 SP) or 0.1 ng/mL PMA and 0.2 μg/mL ionomycin (conditions leading to CD8 SP) as described previously(Wilkinson et al., 2002), and *Twist2* expression was determined by Q-PCR. *Twist2* expression was induced in cultured DP thymocytes activated under conditions that gave rise to either CD4 or CD8 SP cells(Shao et al., 1999; Wilkinson et al., 2002). The induced expression of *Twist2* showed a similar pattern in the early activation phase for

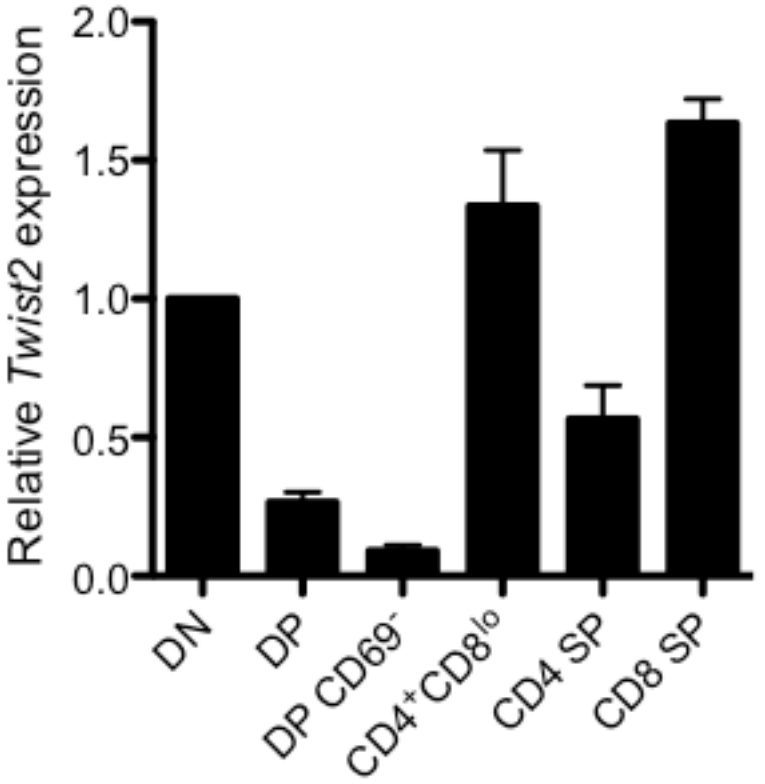


Figure 3. *Twist2* expression is increased in $CD4^{+}CD8^{lo}$ and is 3 fold higher in CD8 SP than in CD4 SP thymocytes.

Twist2 expression was analyzed by quantitative real-time Q-PCR in the indicated thymocyte subsets from C57BL/6 mice. Results are presented relative to GAPDH expression.

up to 3h under both conditions, but showed distinct patterns at the final evaluation time point, similar to its expression in CD4 and CD8 SP thymocytes (**Figure 4A**). Twist2 expression was similarly increased in protein level when activated under the condition leading to CD8 SP differentiation (**Figure 4B**). All these results suggest that Twist2 is upregulated by TCR stimuli at both transcriptional and translational level.

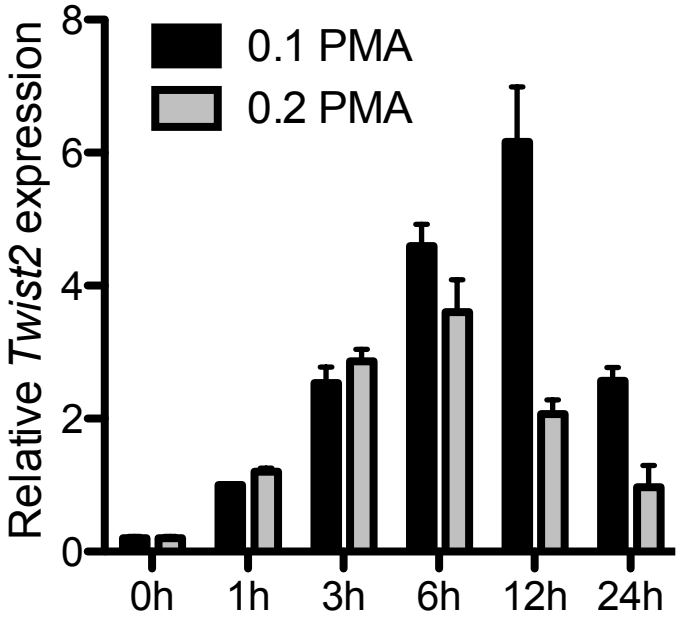
Twist2 interacts with the Runx complex in a CD8 SP differentiation specific manner

On the basis of the differential expression of *Twist2* between CD4 and CD8 lineage cells, I investigated the possible roles of Twist2 during CD4/CD8 thymocyte lineage commitment. It was reported that Runx3 plays an important role in CD8 SP differentiation by repressing ThPOK expression(Setoguchi et al., 2008). Also, Previous studies have reported that Runx proteins and Twist2 interact(Pham et al., 2012; Sharabi et al., 2008; Yang et al., 2011), and I found that Twist2 interacts with Runx3 as shown by co-immunoprecipitation (Co-IP) analysis (**Figure 5**). I also found that Twist2 overexpression in the DP thymoma L7 cell line could downregulate *ThPOK* expression (**Figure 6**). It has been reported that Runx complexes (heterodimers of Cbfβ and Runx1 or Runx3) regulate ThPOK expression by binding to the RBS1 and RBS2

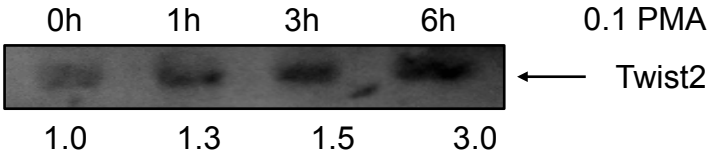
Figure 4. Twist2 expression is induced by TCR signaling and the amount and duration of its expression is greater in CD8 SP induced signal.

(A) Thymocytes from Tcra$^{-/-}$ mice were activated with 0.2 ng/mL or 0.1 ng/mL PMA and 0.2 μg/mL ionomycin for the indicated time periods. Twist2 expression was determined by Q-PCR. (B) Thymocytes from Tcra$^{-/-}$ mice were activated with 0.1 ng/mL PMA and 0.2 μg/mL ionomycin for the indicated times. Nuclear extracts were prepared from the thymocytes. Twist2 expression was determined by western hybridization using anti-Twist2 antibodies.

A


Relative Twist2 expression
0.1 PMA
0.2 PMA
0
2
4
6
8
0h
1h
3h
6h
12h
24h

B


0h
1h
3h
6h
0.1 PMA
Twist2
1.0
1.3
1.5
3.0

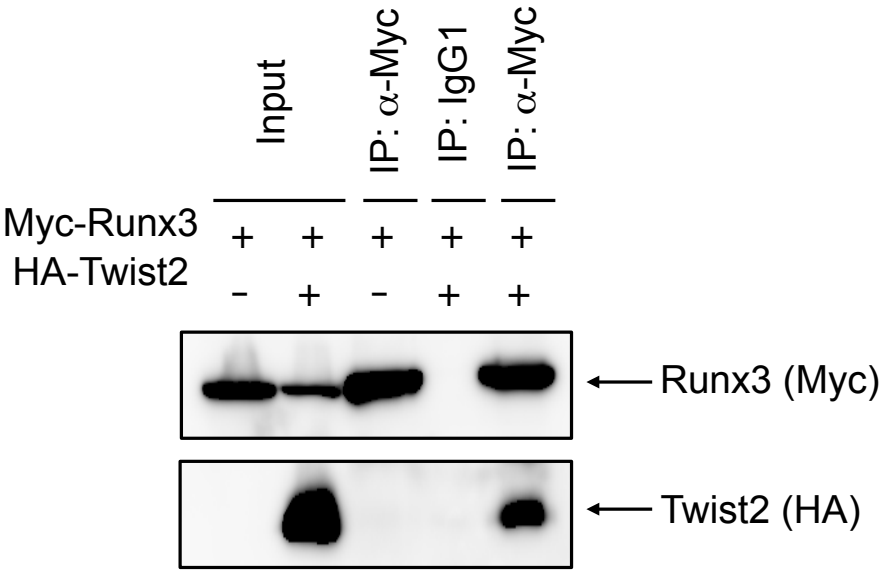
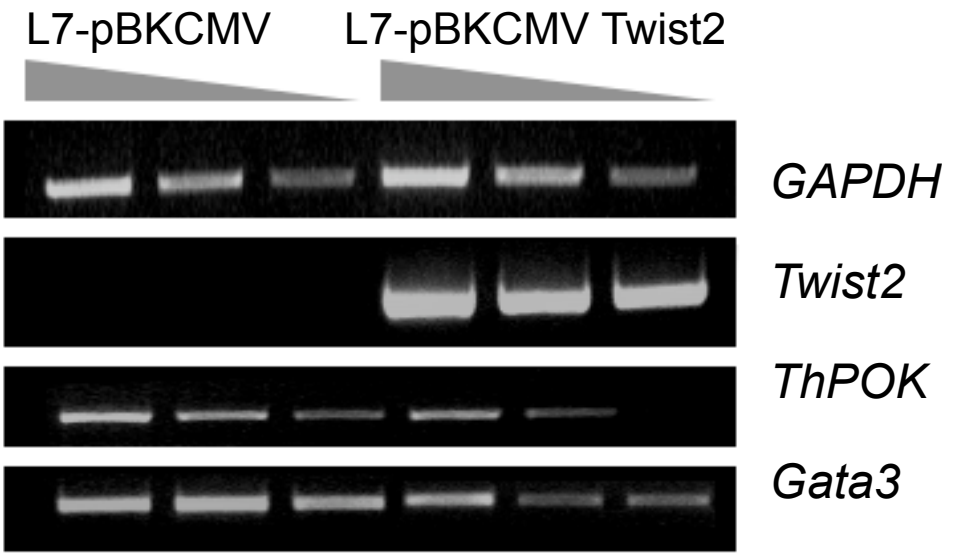


Figure 5. Twist2 interacts with Runx3.

Myc-tagged Runx3 with or without HA-tagged Twist2 was transfected into 293T cells. Protein extracts were immunoprecipitated by using anti-Myc antibodies. Western hybridization was conducted by using anti-Myc and anti-HA antibodies.

Figure 6. Twist2 represses the expression of ThPOK and Gata3 which are important for differentiation of the CD4 SP lineage.

The Twist2-expressing vector was transfected into L7 thymoma cells by electroporation. The expression of *GAPDH, Twist2, ThPOK*, and *Gata3* were determined by semi-quantitative RT-PCR. The data is representative of at least 3 independent experiments.


L7-pBKCMV
L7-pBKCMV Twist2
GAPDH
Twist2
ThPOK
Gata3

regulatory elements of the *ThPOK* gene(Setoguchi et al., 2008) regardless of the differentiation stage. RBS1 is essential for Runx complex-mediated suppression of ThPOK as a silencer, whereas RBS2 is known to be an enhancer. Interestingly, Twist2 could bind to the RBS1 region of *ThPOK*, but not to RBS2 based on a chromatin immunoprecipitation (ChIP) assay against chromatin-protein complexes from $CD4^{+}CD8^{lo}$ thymocytes (**Figures 7A and 7B**). It is to be noted that the interaction of Twist2 to the RBS1 region was nearly lost in $CD4^{+}CD8^{lo}$ thymocytes from β2m-deficient mice, in which CD8 SP differentiation is impaired (**Figure 7C**). Conversely, the interaction was strongly enhanced in cells from *MHC class II*$^{-/-}$ mice. Previous studies have reported that some important regions are located on the ThPOK silencer region (**Figure 2**). To identify where Twist2 binds, a biotinylated-DNA pull-down assay was performed using biotinylated oligomers corresponding to NF-κB and the E box region and Runx-binding region of RBS1 (**Figure 8A**). As a result, I found that Twist2 was specifically bound to the Runx-binding region, but not to NF-κB and the E box region (**Figure 8B**). To be noted is that Twist2 could bind to the Runx site only if both Twist2 and Runx3 were overexpressed. Almost no binding of Twist2 to Runx site was observed without concurrent expression of Runx3 (**Figure 8B**).

To test which domain of Twist2 interacts with Runx complex, I performed Duolink analysis and Co-IP. In Duolink assay, the red fluorescence signal indicated that two proteins are interacting or lie adjacent. Twist2 and Runx3

Figure 7. Twist2 binds to the RBS1 region of ThPOK, but not to RBS2.

(A) ChIP assay was conducted with total thymocytes from Twist2 transgenic mice. Q-PCR analysis was conducted against RBS1, RBS2 and UP1 regions. (B) ChIP assay was conducted with total thymocytes from C57BL/6 wild-type mice. PCR analysis was conducted against the RBS1, RBS2, and Gapdh intron (negative control) region of ThPOK. (C) $CD4^{+}CD8^{lo}$ thymocytes were sorted from the indicated mice by flow cytometry, followed by ChIP with anti-Twist2 antibodies. PCR analysis was conducted against the RBS1 region of ThPOK.

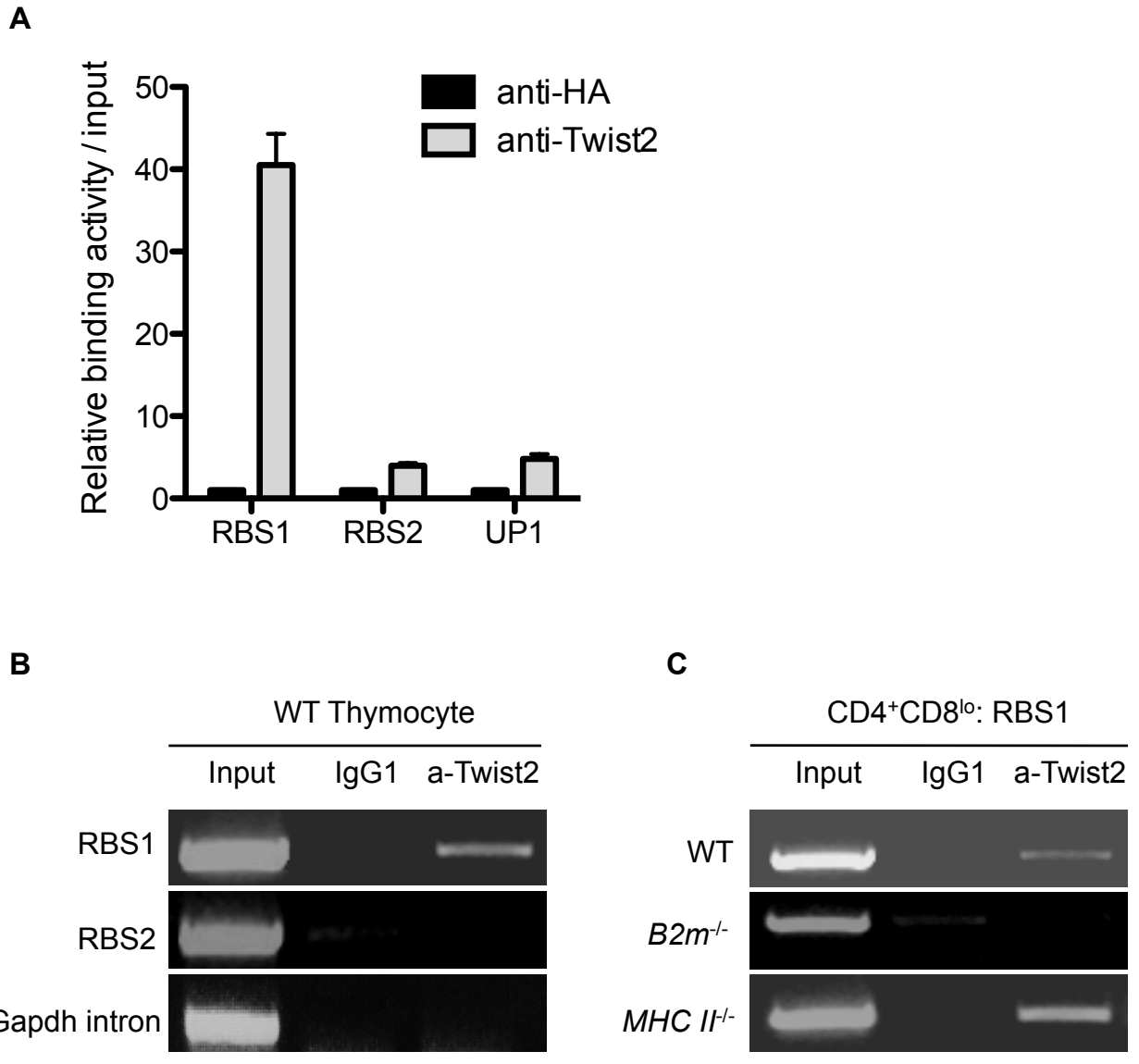
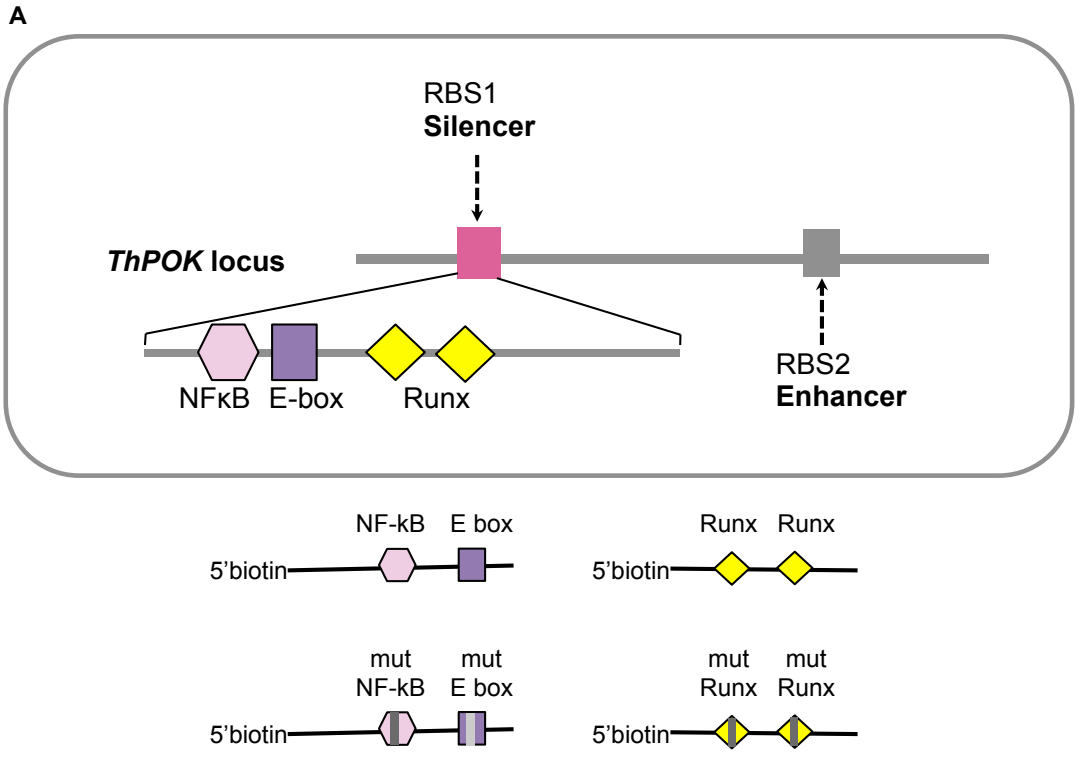

A
Relative binding activity / input
50
40
30
20
10
0
anti-HA
anti-Twist2
RBS1
RBS2
UP1
B
WT Thymocyte
Input
IgG1
a-Twist2
RBS1
RBS2
Gapdh intron
C
CD4+CD8lo: RBS1
Input
IgG1
a-Twist2
WT
B2m-/-
MHC II-/-

Figure 8. Twist2 specifically binds to the Runx sites.

(A) Schematic diagram of position of cis-elements and oligomers used for biotinylated-DNA pull-down assay. (B) Biotinylated-DNA pull-down assay was conducted with biotinylated oligonucleotides, nuclear extracts from 293T cells transfected with HA-Twist2 and myc-Runx3 expressing vectors (upper panel) or only HA-Twist2 expressing vector as a negative control (lower panel), and streptavidin-coated magnetic beads. Pull-downed proteins were analyzed by western hybridization.


A
RBS1
Silencer
ThPOK locus
NFκB
E-box
Runx
RBS2
Enhancer
NF-kB
E box
5'biotin
Runx
Runx
5'biotin
mut
NF-kB
mut
E box
5'biotin
mut
Runx
mut
Runx
5'biotin

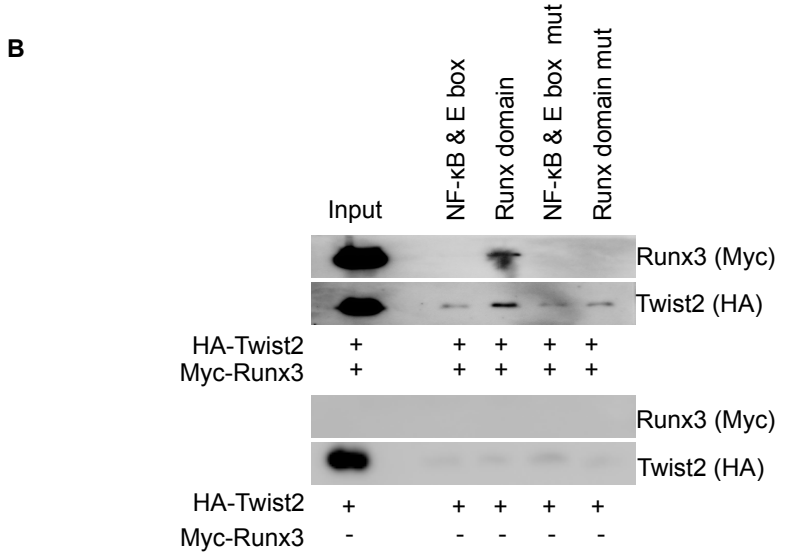
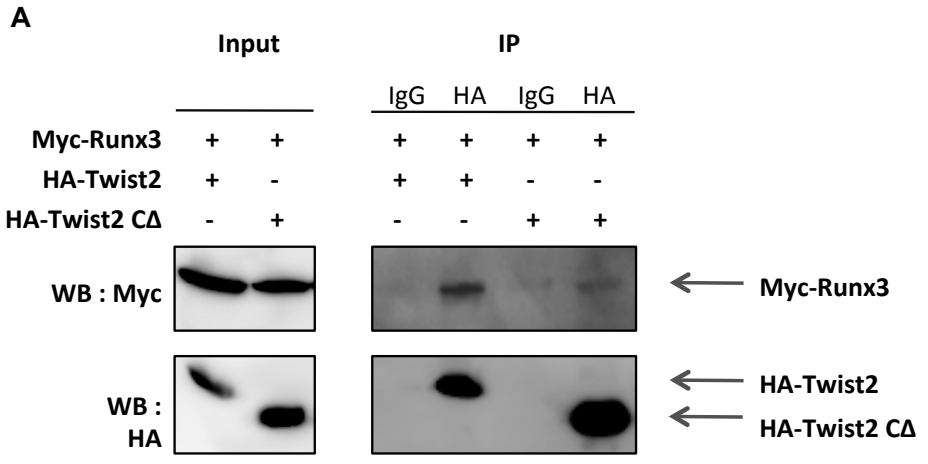
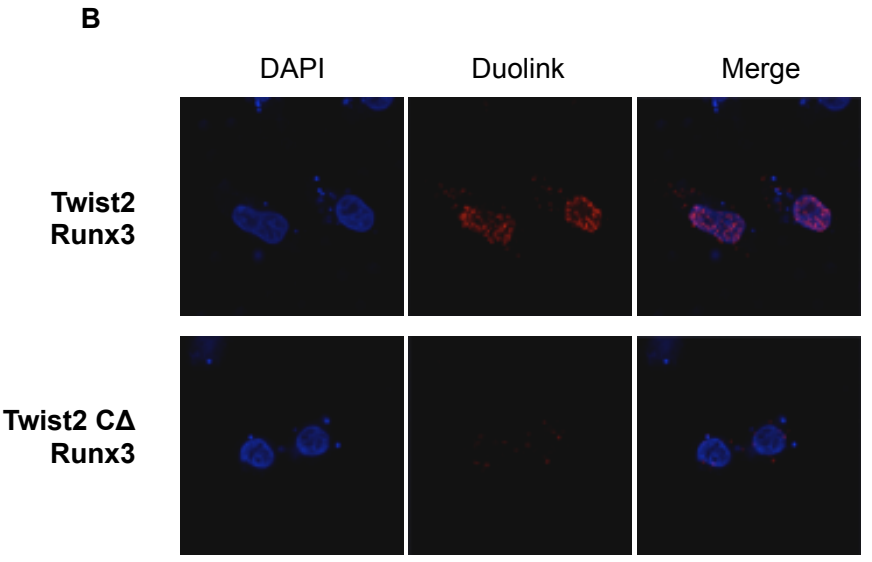

B
Input
NF-κB & E box
Runx domain
NF-κB & E box mut
Runx domain mut
Runx3 (Myc)
Twist2 (HA)
HA-Twist2
Myc-Runx3
Runx3 (Myc)
Twist2 (HA)
HA-Twist2
Myc-Runx3

Figure 9. The interaction between Twist2 and Runx3 is dependent on the Twist box domain.

(A) Myc-tagged Runx3 with HA-tagged Twist2 or HA-tagged Twist2CΔ (Twist box deletion form) was transfected into 293T cells. Cell extracts were immunoprecipitated by using anti-HA or IgG antibodies. Western hybridization was conducted by using anti-Myc and anti-HA antibodies. (B) For Duolink in situ PLA analysis, HeLa cells transfected with the indicated expression vectors with anti-Myc/anti-HA followed by the addition of PLA probes. The fluorescence signal was measured by confocal microscopy.


A
Input
IP
IgG
HA
IgG
HA
Myc-Runx3
HA-Twist2
HA-Twist2 CΔ
WB : Myc
WB : HA
Myc-Runx3
HA-Twist2
HA-Twist2 CΔ


B
DAPI
Duolink
Merge
Twist2 Runx3
Twist2 CΔ Runx3

are immunoprecipitated together in Co-IP and red fluorescence signal was detected in Duolink assay when Twist2 and Runx3 are overexpressed. However, no signal was detected in two experimental systems of Twist2 carboxyl terminal deletion form and Runx3 overexpression condition. Therefore, Twist2 interaction with Runx3 is dependent on Twist2 carboxyl-terminal Twist-box (**Figure 9**). Next, I investigated this interaction between Twist2 and Runx3 or Cbfβ in distinct thymocyte subsets by using Duolink analysis. Importantly, Twist2 and Runx complex interaction was specific only in CD8 SP thymocytes and no significant interaction signal could be observed in CD8 SP thymocytes from *Twist2* cKO mice (**Figure 10**). Furthermore, this interaction was more pronounced in $CD4^{+}CD8^{lo}$ thymocytes from *MHC class II*$^{-/-}$ mice compared with those from *B2m*$^{-/-}$ mice (**Figure 11**). These results indicated that Twist2 and Runx complex interaction plays distinct roles in the critical step of lineage differentiation. It has been reported that the Runx complex binds persistently to the RBS1 region of *ThPOK* in thymocyte subsets, regardless of their capability to differentiate into the CD4 or CD8 SP lineage(Setoguchi et al., 2008). Thus, it has been postulated that some other factor(s) would act to resolve the distinct suppressive activity of the Runx complex on *ThPOK* between CD4 and CD8 SP cells. These results suggest that Twist2 interacts with the Runx complex to suppress the expression of *ThPOK* in a CD8 SP lineage-specific manner.

Figure 10. Twist2 interacts with the Runx complex in CD8 SP.

(A) For Duolink *in situ* PLA analysis, CD4 SP and CD8 SP thymocytes of C57BL/6 and Twist cKO mice were incubated with anti-Twist2 and anti-Cbfβ antibodies, followed by the addition of PLA probes. The fluorescence signal was measured by confocal microscopy. (B) Statistical analysis of Duolink data.

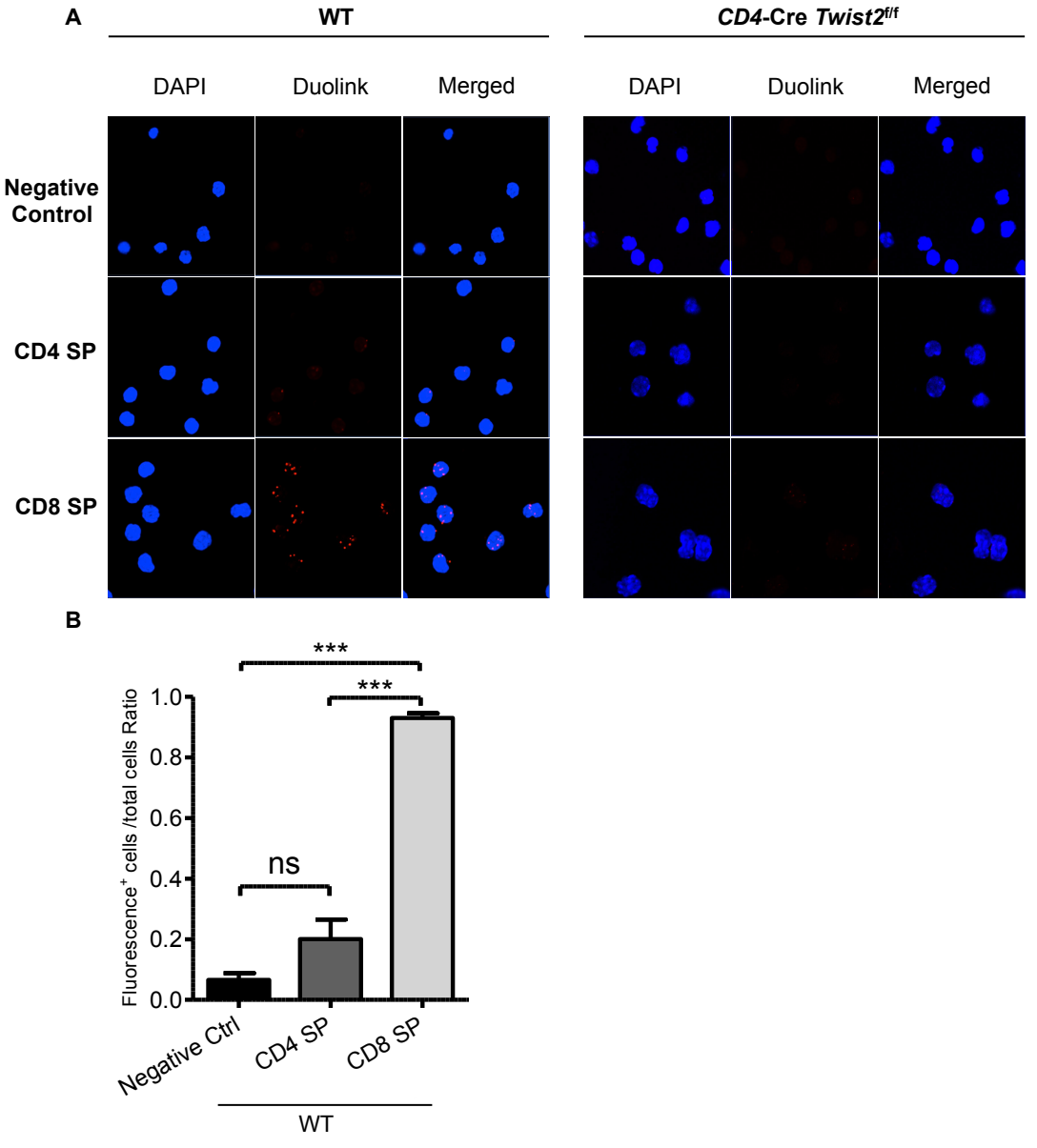
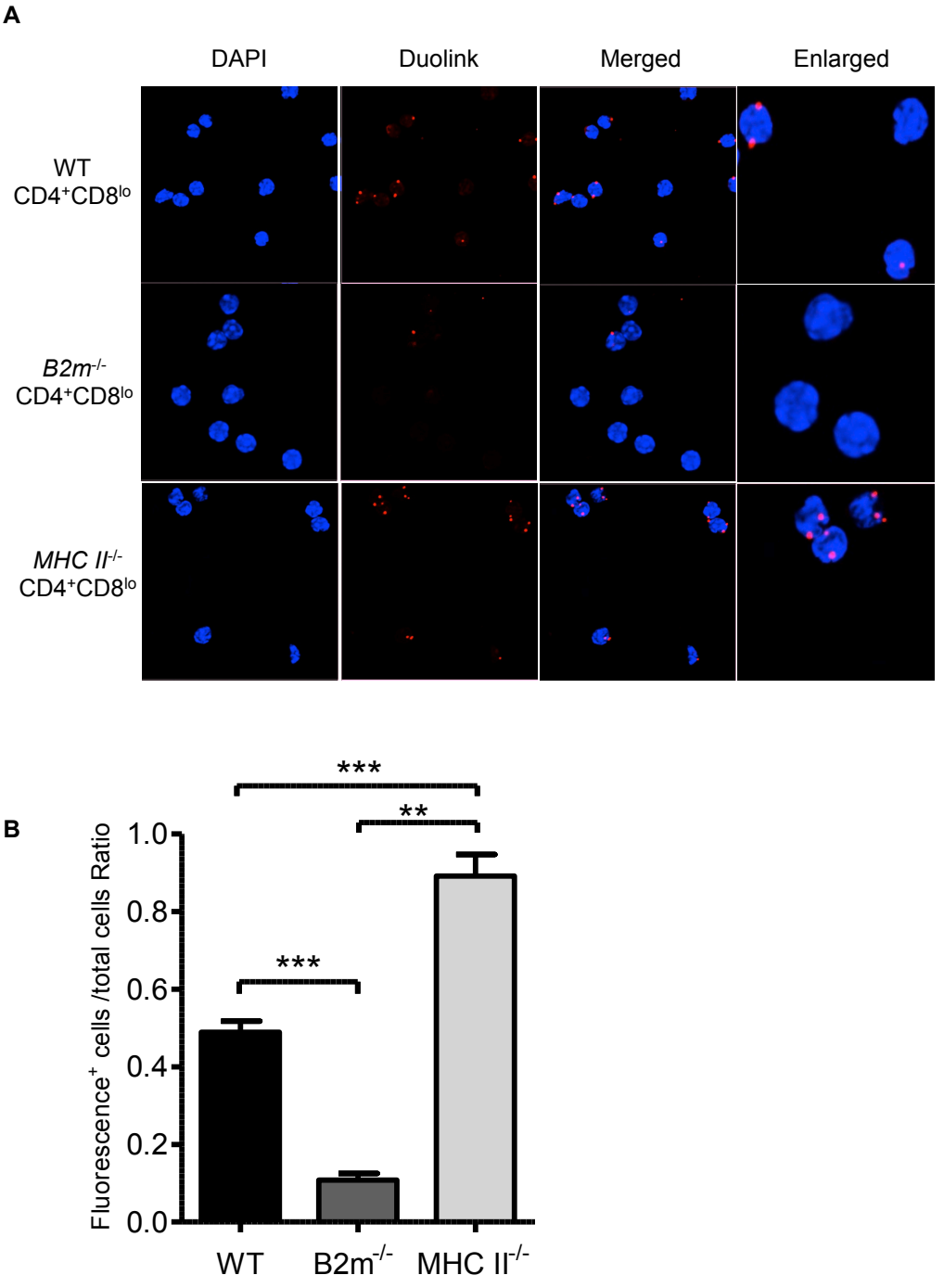

A
WT
CD4-Cre Twist2f/f
DAPI
Duolink
Merged
DAPI
Duolink
Merged
Negative Control
CD4 SP
CD8 SP
B
***
***
ns
Fluorescence+ cells /total cells Ratio
1.0
0.8
0.6
0.4
0.2
0.0
Negative Ctrl
CD4 SP
CD8 SP
WT

Figure 11. Twist2 preferentially interacts with the Runx complex in a CD8 SP differentiation-specific manner.

(A) For Duolink *in situ* PLA analysis, $CD4^{+}CD8^{lo}$ thymocytes of indicated mice were incubated with anti-Twist2 and anti-Cbfβ antibodies, followed by the addition of PLA probes. The fluorescence signal was measured by confocal microscopy. (B) Statistical analysis of Duolink data.


A
DAPI
Duolink
Merged
Enlarged
WT
CD4+CD8lo
B2m-/-
CD4+CD8lo
MHC II-/-
CD4+CD8lo
B
***
**
***
Fluorescence+ cells /total cells Ratio
1.0
0.8
0.6
0.4
0.2
0.0
WT
B2m-/-
MHC II-/-

Twist2 transgenic mice show a decreased ratio of CD4/CD8 subsets and suppressed ThPOK expression

To investigate the function of Twist2, transgenic mice expressing Twist2 under the *lck*-proximal promoter were generated. Intracellular staining assay suggested that the ectopic Twist2 expression was slightly increased in DP thymocytes, significantly increased in $CD4^{+}CD8^{lo}$ population, and unchanged in CD8 SP thymocytes (**Figure 12**). This suggests that transgenic Twist2 protein expression conforms to the general *lck*-proximal promoter pattern. Interestingly, the thymi of *Twist2* transgenic mice were visibly reduced in size, and the total numbers of thymocytes were consistently reduced (**Figure 13A**). The reduced thymic cellularity was accompanied by the increased relative ratio of DN thymocytes (**Figure 13B**), which was considered to be due to impaired DN3 to DN4 transition in the *Twist2* transgenic mice (**Figure 14**). In addition, the ratio of $TCR\beta^{+}CD24^{lo}$ CD4/CD8 SP thymocytes was significantly decreased from 2.18 ± 0.08 (mean ± standard error of the mean [SEM]) in non-transgenic littermate control (NLC) mice to 1.48 ± 0.12 in *Twist2* transgenic mice (**Figure 13C**). In the periphery, the number of total splenocytes was generally decreased in *Twist2* transgenic mice, as for the thymocytes, with a consistent decrease in the ratio of CD4/CD8 SP splenocytes (**Figure 15**).

Twist2 transgenic mice showed significantly reduced *ThPOK* expression in

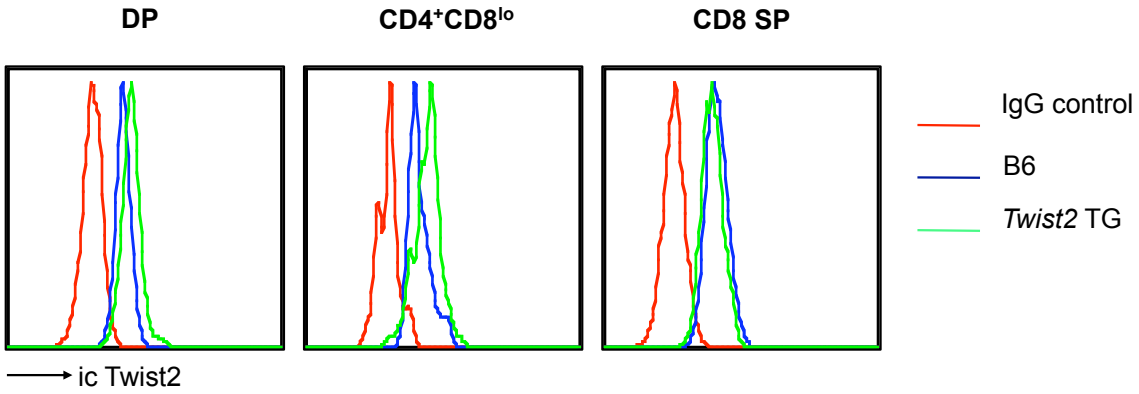
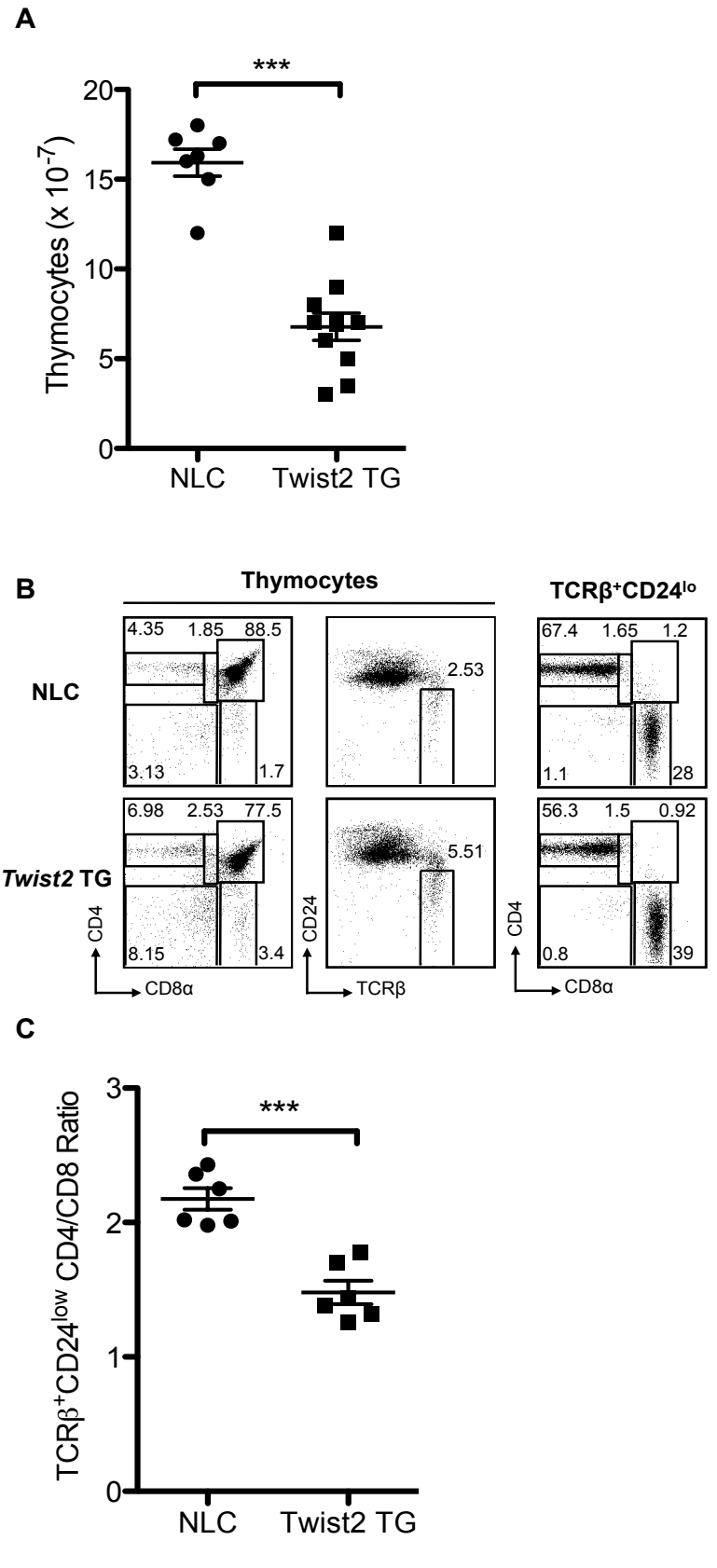


Figure 12. In Twist transgenic mice, ectopic Twist2 expression is slightly increased in DP thymocytes and is more increased in CD4+CD8lo population, but is unchanged in CD8 SP thymocytes.

Ectopic Twist2 expression was detected by intracellular staining assay using anti-Twist2 antibodies.

Figure 13. Twist2 transgenic mice exhibit a reduced ratio of CD4/CD8 subsets.

(A) Numbers of thymocytes from NLC and Twist2 transgenic mice. Each dot indicates an individual mouse; the horizontal lines represent the mean with SEM. ***P < 0.0001. (B) Thymocyte differentiation profiles based on the indicated surface markers from NLC and Twist2 transgenic mice. (C) The ratio of TCRβ+CD24lo CD4 SP cells to CD8 SP cells from NLC and Twist2 transgenic mice. Each symbol indicates an individual mouse; the horizontal lines represent the mean with SEM. ***P < 0.0001 (Student's t-test).


A
***
Thymocytes (x 10^-7)
20
15
10
5
0
NLC
Twist2 TG
B
Thymocytes
TCRβ+CD24lo
NLC
Twist2 TG
4.35
1.85
88.5
3.13
1.7
2.53
67.4
1.65
1.2
1.1
28
6.98
2.53
77.5
8.15
3.4
5.51
56.3
1.5
0.92
0.8
39
CD4
CD8α
CD24
TCRβ
C
***
TCRβ+CD24low CD4/CD8 Ratio
3
2
1
0
NLC
Twist2 TG

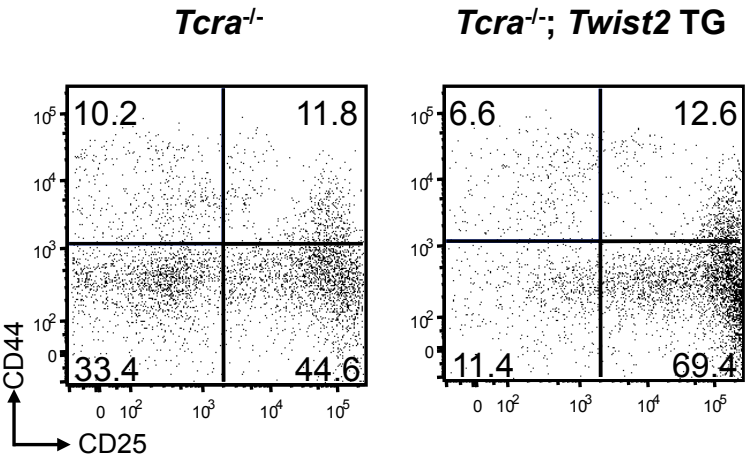


Figure 14. Twist2 transgenic mice result in an impaired DN3 to DN4 transition.

Thymocytes from *Tcra*$^{-/-}$ mice and *Tcra*$^{-/-}$ *Twist2* transgenic mice were stained with fluorochrome-conjugated anti-CD4, anti-CD8α, anti-CD25, and anti-CD44 antibodies, and then analyzed by flow cytometry.

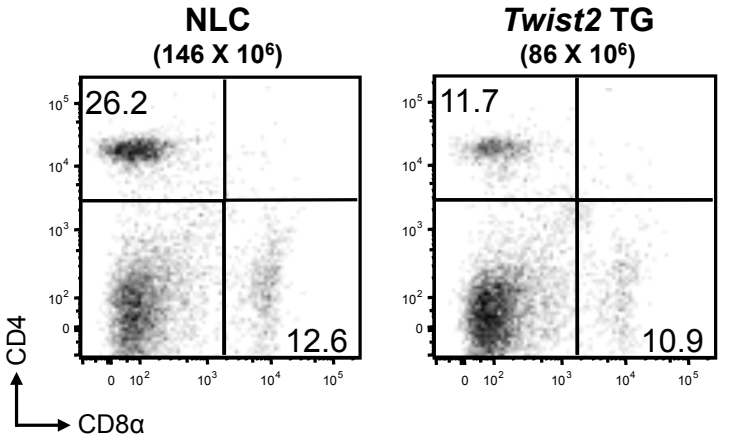


Figure 15. Twist2 transgenic mice show altered CD4/CD8 differentiation profile in splenocytes.

Splenocytes from NLC and *Twist2* transgenic mice were stained anti-CD4, anti-CD8α, and anti-TCRb and analyzed by flow cytometry.

$CD4^+CD8^{lo}$ thymocytes (**Figure 16A**). When I examined ThPOK expression by analyzing the expression of GFP under the control of the *ThPOK* promoter in *ThPOK*-GFP and *Twist2* transgenic mice, the GFP level was significantly reduced in $CD4^+CD8^{lo}$ thymocytes from *ThPOK*-GFP and *Twist2* transgenic mice compared with those from *ThPOK*-GFP mice(Setoguchi et al., 2008) (**Figure 16B**). The marked change in the GFP level in DN thymocytes was mainly due to the lack of mature $CD24^{lo}$ cells (**Figure 17**).

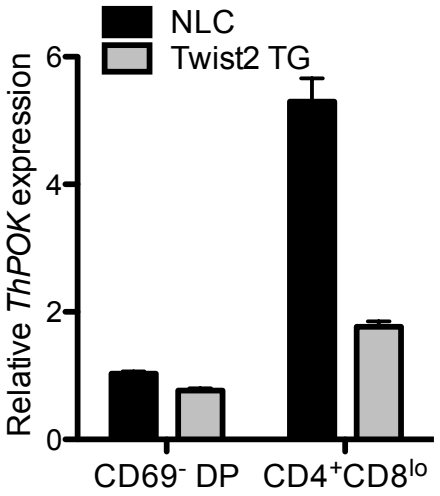
Enhanced CD4 repression in DP thymocytes by Runx3 and Twist2

To investigate the effect of the interaction between Runx3 and Twist2 *in vivo*, I produced mice double transgenic for *Runx3* and *Twist2*, and examined their thymocyte differentiation profiles. As Runx3 binds to the silencer regions of both *CD4* and *ThPOK* and suppresses the expression of those genes, *Runx3* transgenic mice display biased CD4/CD8 lineage differentiation(Hayashi et al., 2001; Kohu et al., 2005). In *Runx3* and *Twist2* double transgenic mice, the DP population was more severely reduced compared with NLC and *Runx3* transgenic mice (**Figure 18**). When $TCR\beta^+CD24^{lo}$ thymocytes were electronically gated, the impairment in CD4 SP development was more pronounced in *Runx3* and *Twist2* double transgenic mice than in *Runx3* transgenic mice. Consistent results were observed for splenocytes (**Figure 18**).

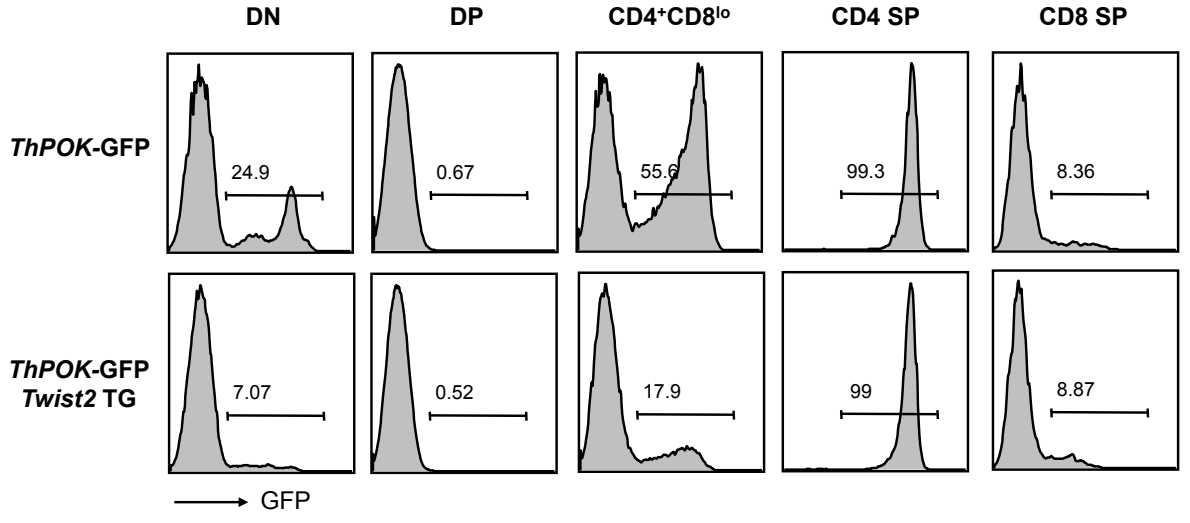
Figure 16. ThPOK expression is reduced in Twist2 transgenic mice.

(A) *ThPOK* expression was determined by Q-PCR in $CD69^-$ DP thymocytes and $CD4^+CD8^{lo}$ thymocytes from NLC and Twist2 transgenic mice sorted by flow cytometry. (B) ThPOK expression was determined by the relative fluorescence intensity of GFP from the indicated thymocyte subsets from the indicated mice. The numbers above the bracketed lines represent percent GFP-positive cells.

A


NLC
Twist2 TG
Relative ThPOK expression
6
4
2
0
CD69- DP
CD4+CD8lo

B


DN
DP
CD4+CD8lo
CD4 SP
CD8 SP
ThPOK-GFP
24.9
0.67
55.6
99.3
8.36
ThPOK-GFP
Twist2 TG
7.07
0.52
17.9
99
8.87
GFP

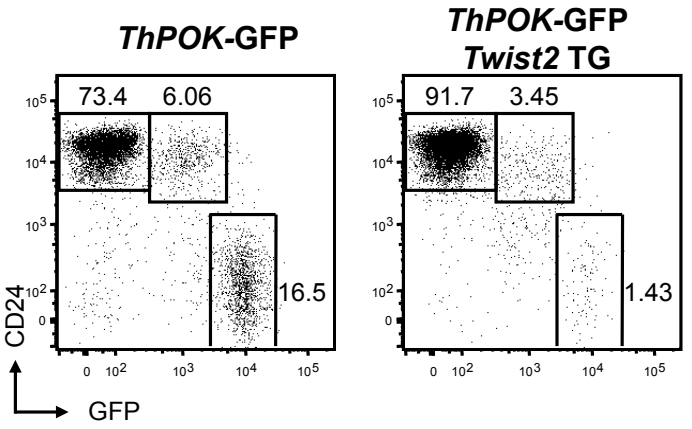
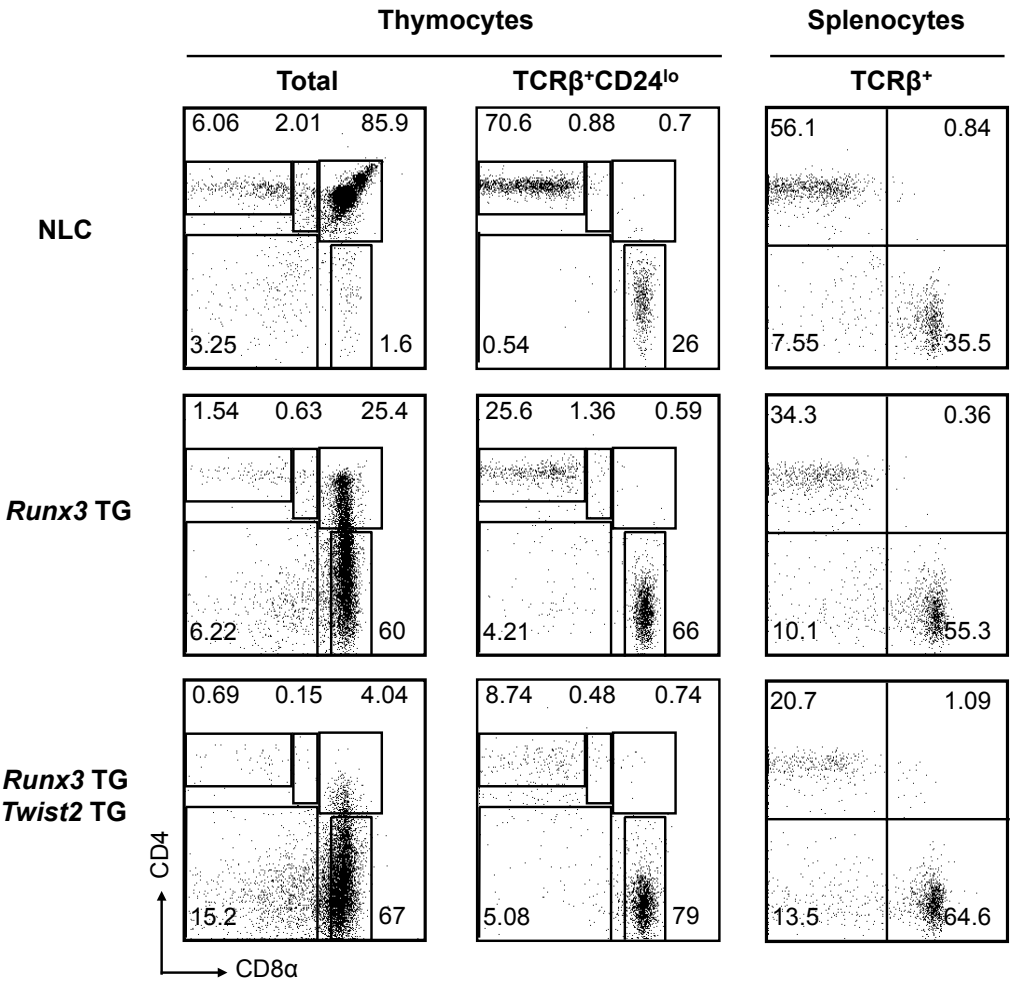


Figure 17. Marked decrease in ThPOK-GFP^{+} DN cells is due to the lack of mature CD24^{lo} cells.

DN cells were electronically gated, and then their CD24 and GFP levels were analyzed.

Figure 18. Analysis of Runx3 and Twist2 transgenic mice by flow cytometry.

CD4 and CD8α expression in total thymocytes (left), $TCR\beta^+$ thymocytes (middle), and $TCR\beta^+$ splenocytes (right) from the NLC, Runx3 transgenic mice, and Runx3 and Twist2 double transgenic mice.


Thymocytes
Splenocytes
Total
TCRβ+CD24lo
TCRβ+
NLC
Runx3 TG
Runx3 TG
Twist2 TG
6.06
2.01
85.9
3.25
1.6
70.6
0.88
0.7
0.54
26
56.1
0.84
7.55
35.5
1.54
0.63
25.4
6.22
60
25.6
1.36
0.59
4.21
66
34.3
0.36
10.1
55.3
0.69
0.15
4.04
15.2
67
8.74
0.48
0.74
5.08
79
20.7
1.09
13.5
64.6
CD4
CD8α

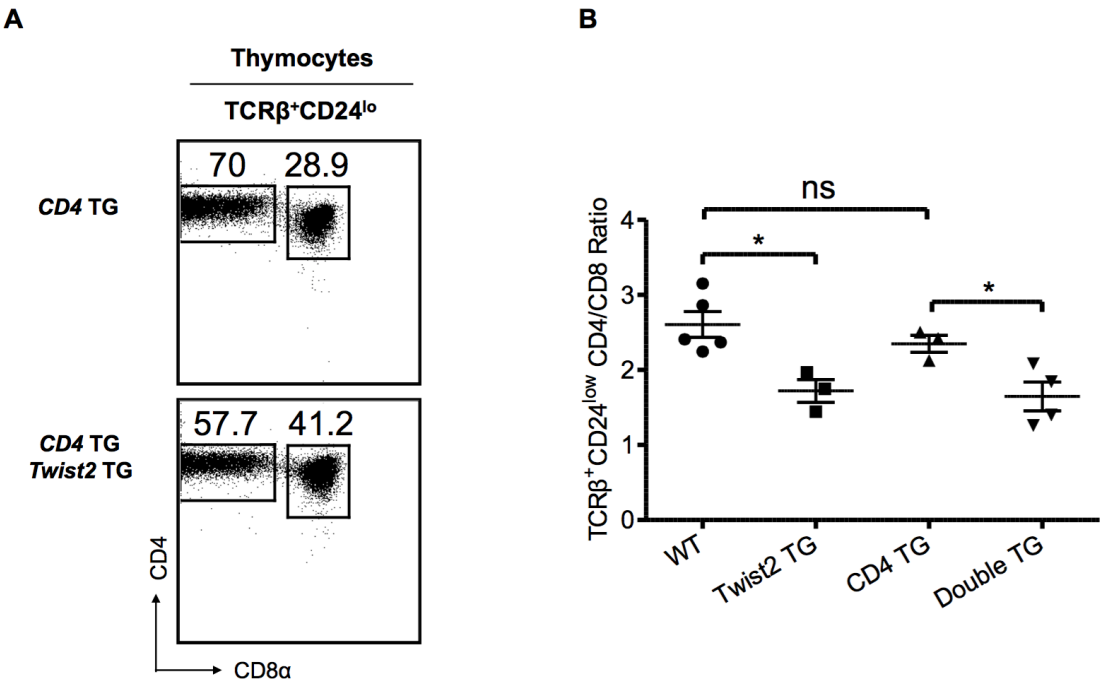
Notably, *Twist2* and *Runx3* double transgenic mice showed more severe reduction in CD4 expression in addition to CD4 SP thymocytes. As the expression level of CD4 itself in DP thymocytes can affect the lineage choice, I investigated whether Twist2 can modulate CD4 expression, and whether continuous expression of CD4 can block the Twist2-induced CD8 SP differentiation.

Reporter assay with *Cd4* promoter, proximal enhancer, silencer, and distal enhancer, and ChIP assay suggested that Twist2 could bind to the *Cd4* proximal enhancer and *Cd4* silencer, and regulate the *Cd4* gene in DP thymocytes (data not shown). This raises a possibility that the aberrant CD4/CD8 lineage differentiation in *Twist2* transgenic mice may result from the downregulation of CD4 as observed in *Runx3* transgenic mice. Though the reduction in CD4 expression in DP thymocytes was observed in the *Twist2* transgenic mouse line presented here, the downregulation was modest. Furthermore, the decreased CD4/CD8 SP ratio was also observed in another *Twist2* transgenic line without any significant change in CD4 level (data not shown). In *Twist2*-transfected thymoma cell lines, *ThPOK* could be downregulated regardless of TCR stimuli (**Figure 6**).

In *Twist2* and *CD4* double transgenic mice, the electronically gated $TCR\beta^{+}CD24^{lo}$ cells showed similar decrease in CD4/CD8 SP ratio to those from *Twist2* transgenic mice (**Figure 19**). Also, I tested whether Twist2 can affect MHC class II-restricted cells with constitutive *CD4* transgene. *CD4* transgenic systems of $B2m^{-/-}$ or MHC class II-restricted TCR transgenic mice have mature $CD4^{+}CD8^{+}$ cells with cytotoxic function(Davis et al., 1993).

Figure 19. In Twist2 and CD4 double transgenic mice, the electronically gated TCRβ^{+}CD24^{lo} cells show similar decrease in CD4/CD8 SP ratio to those from Twist2 transgenic mice.

(A) Thymocyte differentiation profiles based on the CD4, CD8, TCRβ, and CD24 surface markers from CD4 tansgenic mice and CD4 and Twist2 double transgenic mice. (B) The ratio of TCRβ^{+}CD24^{lo} CD4/CD8 SP cells from the indicated mice.


A
Thymocytes
TCRβ+CD24lo
CD4 TG
70
28.9
CD4 TG
Twist2 TG
57.7
41.2
CD4
CD8α
B
TCRβ+CD24low CD4/CD8 Ratio
ns
*
*
0
1
2
3
4
WT
Twist2 TG
CD4 TG
Double TG

Likewise, OT2-TCR and *CD4* double transgenic mice have mature $CD4^+CD8^+$ cells. In this system, OT2-TCR, *Twist2* and *CD4* triple transgenic mice have an even greater number of $CD4^+CD8^+$ cells (**Figure 20**). Therefore, the reduction of mature CD4 SP cells in *Twist2* transgenic mice is not likely to be caused by the decreased CD4 expression during DP development, but by the Twist2 acting directly to drive the CD8 SP lineage.

Altered CD4/CD8 SP thymocyte differentiation in *Twist2* conditional knock out mice

As *Twist2* knock out mice show perinatal death and systemic elevation of various cytokines that affect thymocyte development and differentiation(Sosic et al., 2003), I used a *Twist2* conditional knock out (cKO) mouse system to investigate the effect of loss-of-function of Twist2 during thymocyte differentiation. The CD4-specific deletion of *Twist2* showed a slight decrease in total thymic cellularity (**Figure 21A**). Compared with *CD4*-Cre *Twist2*$^{+/+}$ mice, *CD4*-Cre *Twist2*$^{f/f}$ mice showed altered CD4 and CD8 SP differentiation profiles with a decreased percentage of $TCR\beta^+CD24^{lo}$ thymocytes (**Figure 21B**). In addition, the ratio of $TCR\beta^+CD24^{lo}$ CD4 SP to CD8 SP thymocytes was significantly increased from 2.06 ± 0.132 in *CD4*-Cre *Twist2*$^{+/+}$ mice to 3.14 ± 0.174 in *CD4*-Cre *Twist2*$^{f/f}$ mice, which is thought to result from decrease in CD8 SP cells (**Figures 21C** and **21D**).

Figure 20. Mature $CD4^+CD8^+$ cells with cytotoxic function are increased in OT2-TCR, *CD4* and *Twist2* triple transgenic mice compared to the controls.

(A) Flow cytometry analysis of CD4, CD8α, CD24 and Vα2 expression on thymocytes from the indicated mice. (B) Q-PCR analysis of RNA purified from indicated populations of thymocytes from the indicated mice against *Perforin1*.

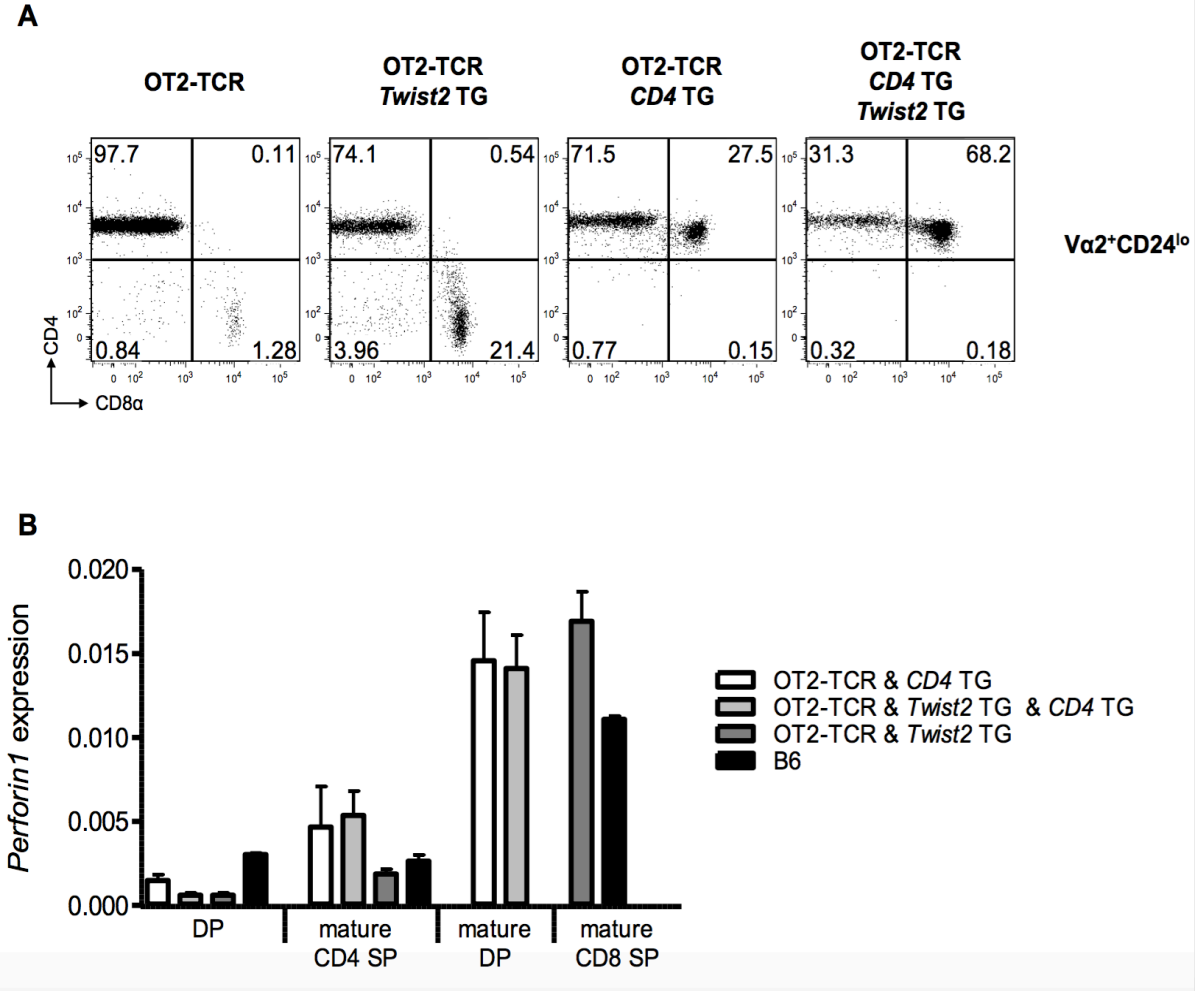
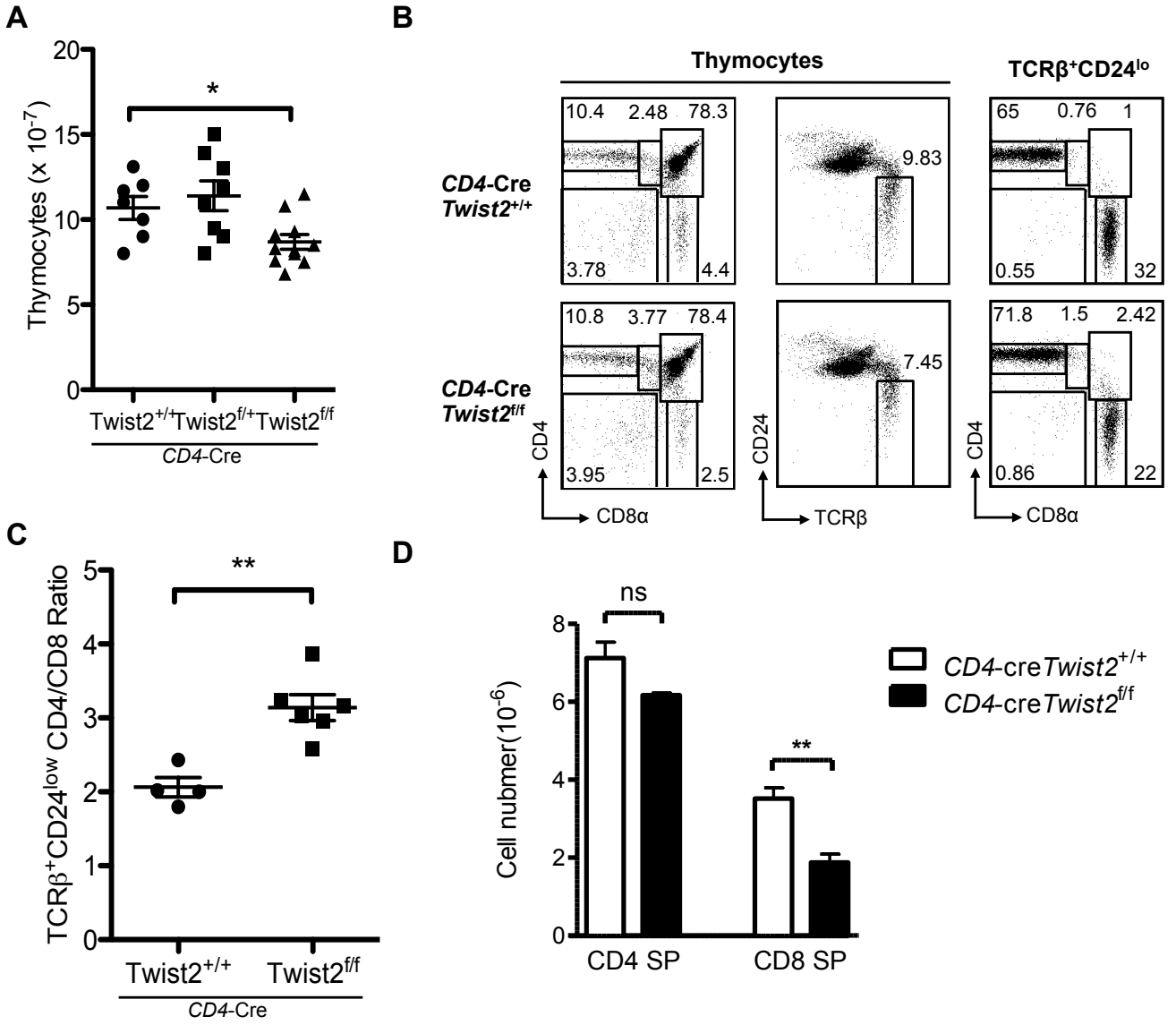

A
OT2-TCR
OT2-TCR
Twist2 TG
OT2-TCR
CD4 TG
OT2-TCR
CD4 TG
Twist2 TG
97.7
0.11
0.84
1.28
74.1
0.54
3.96
21.4
71.5
27.5
0.77
0.15
31.3
68.2
0.32
0.18
CD4
CD8α
Vα2+CD24lo
B
Perforin1 expression
0.020
0.015
0.010
0.005
0.000
DP
mature CD4 SP
mature DP
mature CD8 SP
OT2-TCR & CD4 TG
OT2-TCR & Twist2 TG & CD4 TG
OT2-TCR & Twist2 TG
B6

Figure 21. Thymocyte-specific Twist2 deficiency is associated with an increased ratio of CD4/CD8 subsets.

(A) Numbers of thymocytes from *CD4*-Cre *Twist2*$^{+/+}$, *CD4*-Cre *Twist2*$^{f/+}$ and *CD4*-Cre *Twist2*$^{f/f}$ mice. Each symbol indicates an individual mouse; the horizontal lines represent the mean with SEM. $^{*}P = 0.0180$ (Student's *t*-test). (B) Thymocyte differentiation profiles based on the indicated surface markers from the indicated mice. (C) The ratio of TCRβ^{+}CD24^{lo} CD4/CD8 SP cells from the indicated mice. Each symbol indicates an individual mouse; the horizontal lines represent the mean with SEM. $^{**}P = 0.0021$ (Student's *t*-test). (D) Cell numbers of CD4 SP and CD8 SP from *CD4*-Cre *Twist2*$^{+/+}$ and *CD4*-Cre *Twist2*$^{f/f}$ mice.


A
Thymocytes (x 10-7)
20
15
10
5
0
*
Twist2+/+ Twist2f/+ Twist2f/f
CD4-Cre
B
Thymocytes
TCRβ+CD24lo
CD4-Cre Twist2+/+
CD4-Cre Twist2f/f
10.4 2.48 78.3
3.78 4.4
9.83
65 0.76 1
0.55 32
10.8 3.77 78.4
3.95 2.5
7.45
71.8 1.5 2.42
0.86 22
CD4
CD8α
CD24
TCRβ
C
TCRβ+CD24low CD4/CD8 Ratio
5
4
3
2
1
0
**
Twist2+/+ Twist2f/f
CD4-Cre
D
Cell nubmer(10-6)
8
6
4
2
0
ns
**
CD4 SP
CD8 SP
CD4-creTwist2+/+
CD4-creTwist2f/f

While *Twist2* transgenic mice showed significantly reduced *ThPOK* expression in $CD4^{+}CD8^{lo}$ thymocytes, *Twist2* cKO mice showed increased *ThPOK* expression in the same population (**Figure 22**). There was also a slight increase in *ThPOK* expression in CD8 SP thymocytes. In addition, the *lck*-dependent deletion of *Twist2* increased the number of $TCR\beta^{+}CD24^{lo}$ CD4 SP thymocytes in *Runx3* transgenic mice (**Figure 23**).

Altered CD4/CD8 SP thymocyte differentiation in *Twist2* transgenic mice and *Twist2* cKO mice expressing transgenic TCR

On the basis of the differential expression of Twist2 during CD4 and CD8 SP thymocyte differentiation *in vitro*, and the altered CD4/CD8 SP thymocyte ratio in *Twist2* transgenic mice, I further investigated the effect of Twist2 in TCR transgenic mice. As DO-TCR is restricted to MHC class II molecules, these transgenic mice preferentially generate CD4 SP cells(Hogquist et al., 1994). In $Rag^{-/-}$ DO-TCR and *Twist2* double transgenic mice, the percentage and the cell number of the CD4 SP population was significantly decreased, whereas the percentage and the cell number of the CD8 SP population was increased, resulting in a decreased relative $DO^{+}CD24^{lo}$ CD4/CD8 SP thymocyte ratio (**Figure 24**). In splenocytes, $Rag^{-/-}$ DO-TCR and *Twist2* double transgenic mice showed a consistent decrease in the CD4 SP population and an increase in the CD8 SP population (**Figure 24A**). Both $CD8^{+}$ thymocytes and

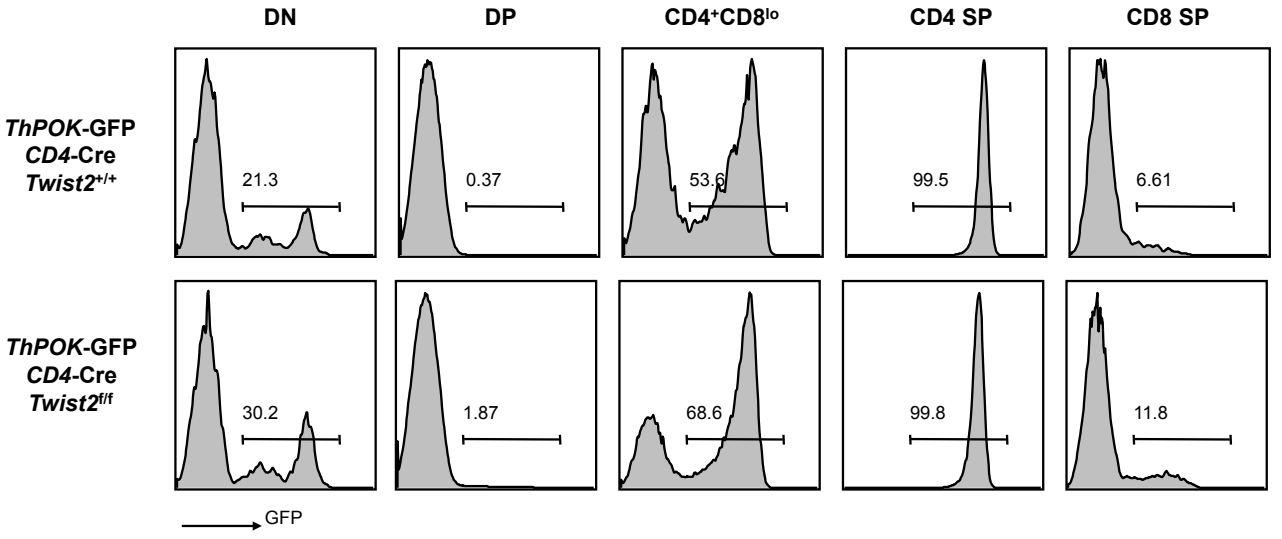


Figure 22. ThPOK expression is increased in Twist2 conditional knock out mice.

ThPOK expression was determined by the relative fluorescence intensity of GFP from the indicated thymocyte subsets from the indicated mice. The numbers above the bracketed lines represent percent GFP-positive cells.

Figure 23. *lck*-dependent deletion of *Twist2* increased the number of TCRβ^{+}CD24^{lo} CD4 SP thymocytes in *Runx3* transgenic mice.

CD4 and CD8α expression in total thymocytes and TCRβ^{+}CD24^{lo} thymocytes from the indicated mice.

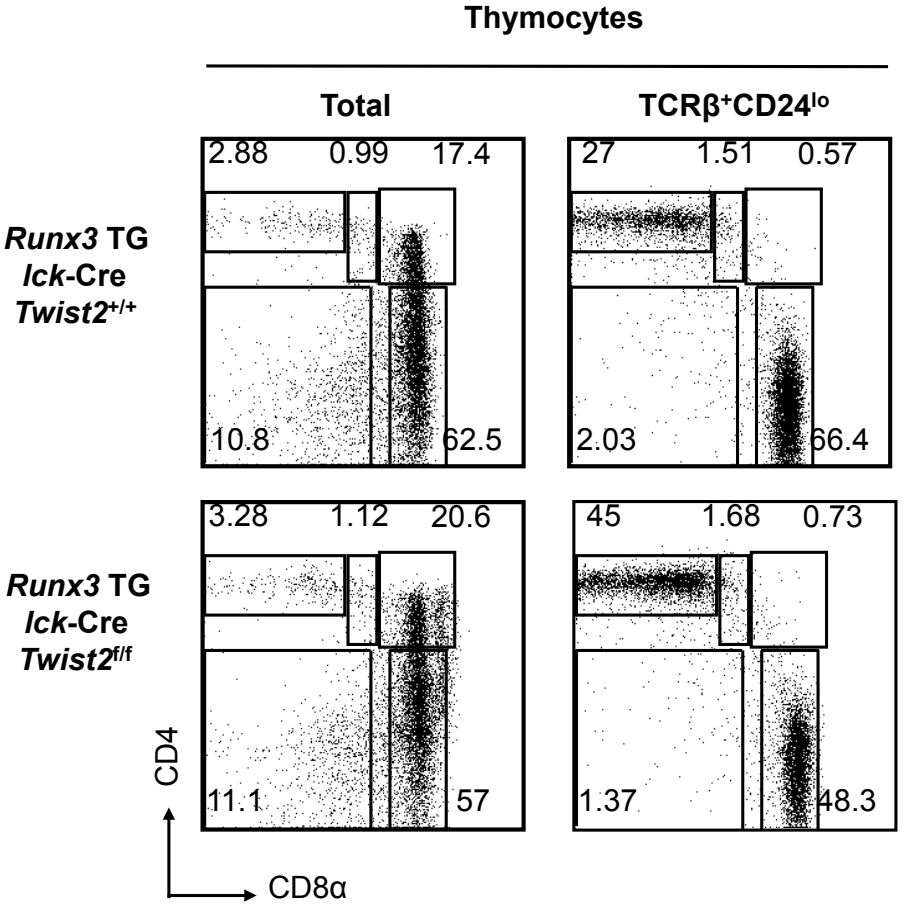
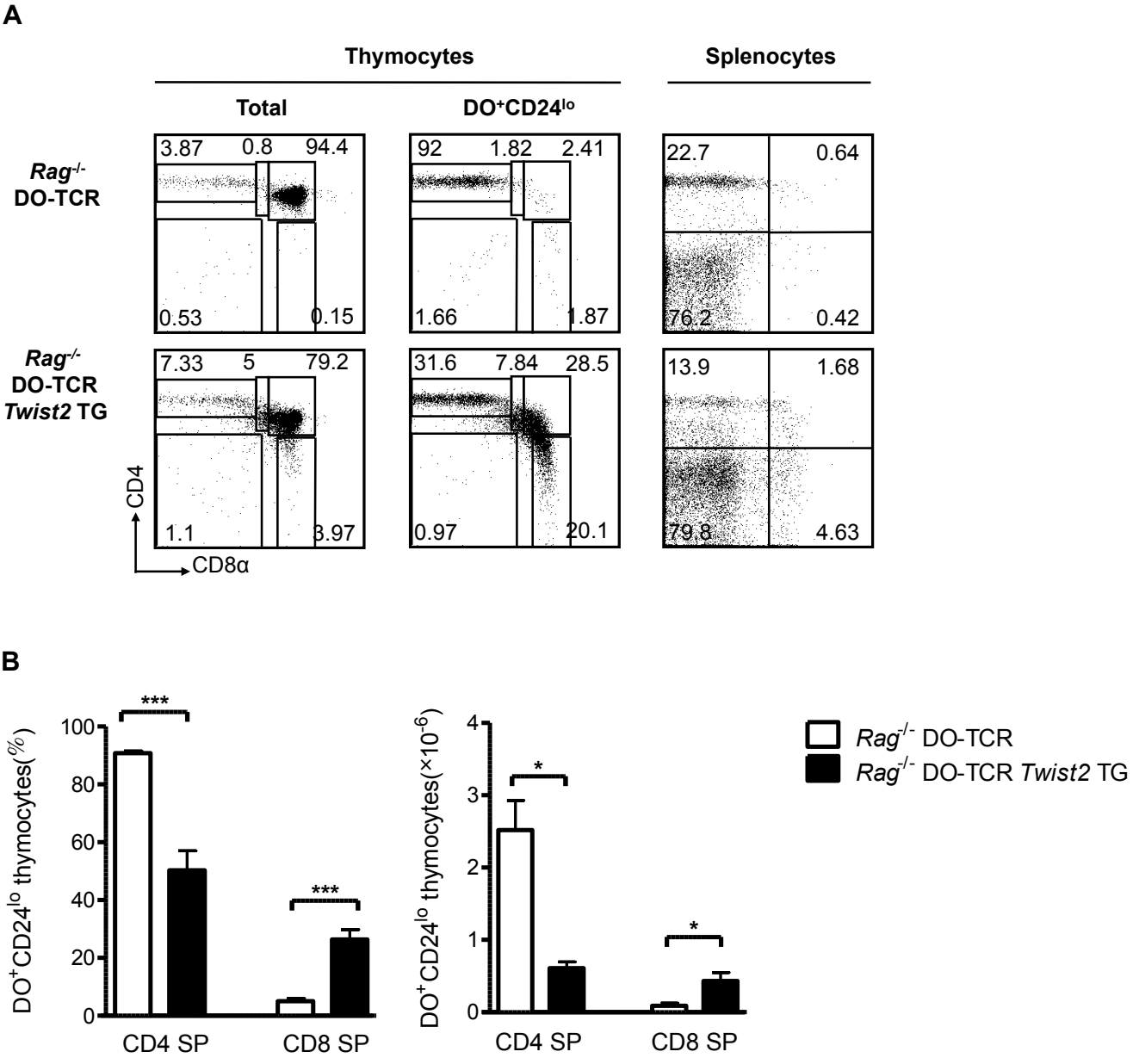

Thymocytes
Total
TCRβ+CD24lo
Runx3 TG
lck-Cre
Twist2+/+
2.88
0.99
17.4
10.8
62.5
27
1.51
0.57
2.03
66.4
Runx3 TG
lck-Cre
Twist2f/f
3.28
1.12
20.6
11.1
57
45
1.68
0.73
1.37
48.3
CD4
CD8α

Figure 24. In *Rag*$^{-/-}$ DO-TCR and Twist2 double transgenic mice, the percentage and the cell number of the CD4 SP population is significantly decreased, whereas the percentage and the cell number of the CD8 SP population is increased.

(A) CD4 and CD8α expression in total thymocytes, DO^{+}CD24^{lo} thymocytes, and splenocytes from *Rag*$^{-/-}$ DO-TCR transgenic mice and *Rag*$^{-/-}$ DO-TCR *Twist2* transgenic mice. (B) Frequency and cell number of mature CD4 SP and CD8 SP thymocytes in *Rag*$^{-/-}$ DO-TCR transgenic mice and *Rag*$^{-/-}$ DO-TCR *Twist2* transgenic mice.


A
Thymocytes
Splenocytes
Total
DO+CD24lo
Rag-/- DO-TCR
Rag-/- DO-TCR Twist2 TG
3.87
0.8
94.4
0.53
0.15
92
1.82
2.41
1.66
1.87
22.7
0.64
76.2
0.42
7.33
5
79.2
1.1
3.97
31.6
7.84
28.5
0.97
20.1
13.9
1.68
79.8
4.63
CD4
CD8α
B
DO+CD24lo thymocytes(%)
DO+CD24lo thymocytes(×10-6)
CD4 SP
CD8 SP
Rag-/- DO-TCR
Rag-/- DO-TCR Twist2 TG

splenocytes have CD8αβ heterodimer phenotype (**Figure 25**). Conversely, when *Twist2* transgenic mice were crossed with *Rag*$^{-/-}$ HY-TCR transgenic mice, in which transgenic TCR is restricted to MHC class I molecules(Kisielow et al., 1988), differentiation into the CD8 SP population was further enhanced in *Rag*$^{-/-}$ HY-TCR and *Twist2* double transgenic mice (**Figure 26A** and **26B**).

To further investigate the loss of Twist2 function during thymocyte differentiation, I generated *Twist2* cKO mice under various TCR transgenic backgrounds. Interestingly, CD8 SP differentiation was significantly perturbed in *Rag*$^{-/-}$ HY-TCR *CD4*-Cre *Twist2*$^{f/f}$ mice, where overall thymocyte cellularity was reduced, and consequently there was a significant reduction in mature CD8 SP cells (**Figures 27A** and **27B**). In contrast, CD4 SP thymocytes were slightly increased in the MHC class II-restricted TCR-transgenic background (**Figure 28**). *Rag*$^{-/-}$ DO-TCR *CD4*-Cre *Twist2*$^{f/f}$ mice showed increased numbers of DO^{+} thymocytes compared with *Rag*$^{-/-}$ DO-TCR *CD4*-Cre *Twist2*$^{+/+}$ control mice, and DO^{+} CD4 SP thymocytes were also slightly increased.

Lineage conversion in *Twist2* transgenic and *Twist2* cKO mice

On the basis of the finding that Twist2 produced MHC class II-restricted CD8 SP cells in *Rag*$^{-/-}$ DO-TCR transgenic mice, I next tested whether Twist2 can generate CD8 SP cells in the absence of MHC class I. Compared with *B2m*$^{-/-}$

Figure 25. $CD4^{low}CD8^{+}$ mature thymocytes and splenocytes in *Rag*$^{-/-}$ DO-TCR and *Twist2* double transgenic mice express CD8αβ heterodimers.

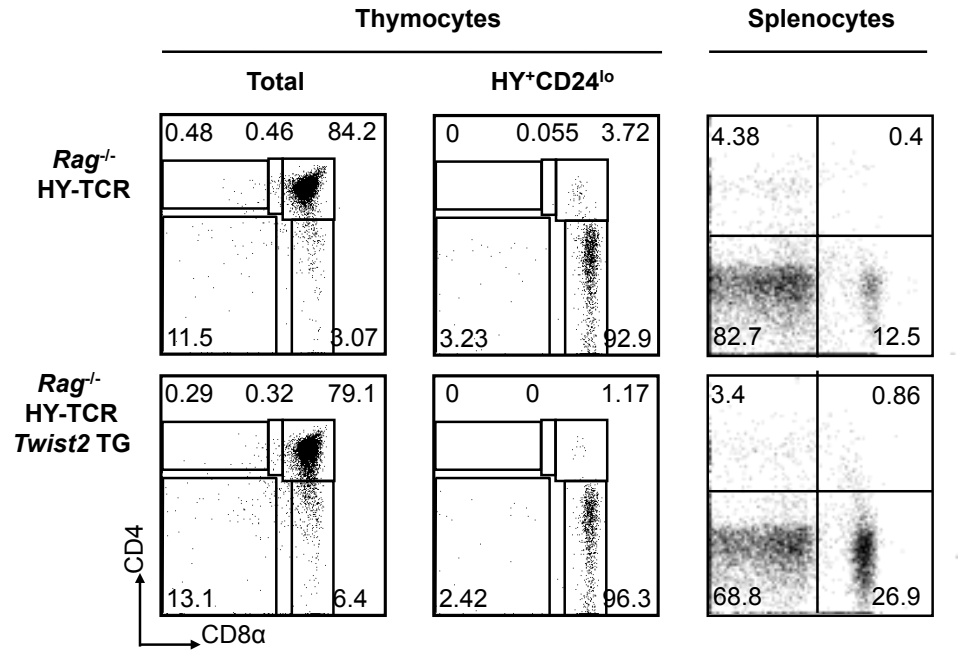
(A) Expression of CD4, CD8α and CD8β by mature $DO^{+}CD24^{lo}$ thymocytes from the indicated mice. (B) Expression of CD4, CD8α and CD8β by splenocytes from the indicated mice.


A
DO+CD24lo
1
2
3
Rag-/-
DO-TCR
5.8
0.94
0.37
Rag-/-
DO-TCR
Twist2 TG
22.8
9.77
4.22
CD4
CD8α
CD8β
CD8α
B
Total
DO+
Rag-/-
DO-TCR
0.42
0
50
0
50
0.42
0
91.4
0
8.57
Rag-/-
DO-TCR
Twist2 TG
2.06
0
82.8
0
17.2
4.64
0
97.2
0
2.78
CD4
CD8α
CD8β
CD8α
CD4
CD8α
CD8β
CD8α

Figure 26. Differentiation into the CD8 SP population is further enhanced in *Rag*$^{-/-}$ HY-TCR and *Twist2* double transgenic mice compared with *Rag*$^{-/-}$ HY-TCR mice.

(A) CD4 and CD8α expression in total thymocytes, HY^{+}CD24^{lo} thymocytes and splenocytes from *Rag*$^{-/-}$ HY-TCR transgenic mice and *Rag*$^{-/-}$ HY-TCR *Twist2* transgenic mice. (B) Frequency of mature CD4 SP and CD8 SP thymocytes in (A).

A


Thymocytes
Splenocytes
Total
HY+CD24lo
Rag-/- HY-TCR
Rag-/- HY-TCR Twist2 TG
0.48 0.46 84.2
11.5 3.07
0 0.055 3.72
3.23 92.9
4.38 0.4
82.7 12.5
0.29 0.32 79.1
13.1 6.4
0 0 1.17
2.42 96.3
3.4 0.86
68.8 26.9
CD4
CD8α

B

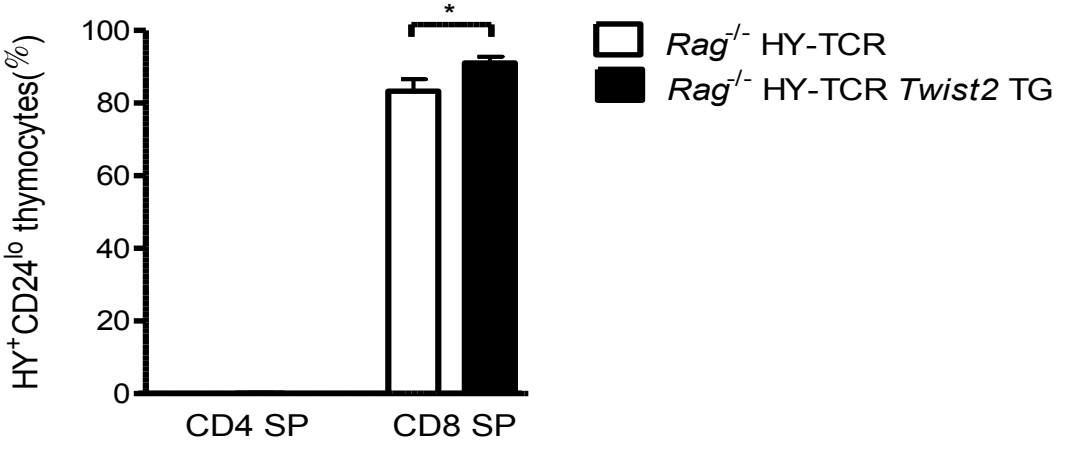

HY+CD24lo thymocytes(%)
100
80
60
40
20
0
*
CD4 SP
CD8 SP
Rag-/- HY-TCR
Rag-/- HY-TCR Twist2 TG

Figure 27. CD8 SP differentiation is significantly perturbed in *Rag*$^{-/-}$ HY-TCR *CD4*-Cre *Twist2*$^{f/f}$ mice compared with control mice.

(A) CD4 and CD8α expression in total thymocytes and HY^{+}CD24^{lo} thymocytes from *Rag*$^{-/-}$ HY-TCR *CD4*-Cre *Twist2*$^{+/+}$ mice and *Rag*$^{-/-}$ HY-TCR *CD4*-Cre *Twist2*$^{f/f}$ mice. (B) Frequency and cell number of mature CD4 SP and CD8 SP thymocytes in *Rag*$^{-/-}$ HY-TCR *CD4*-Cre *Twist2*$^{+/+}$ mice and *Rag*$^{-/-}$ HY-TCR *CD4*-Cre *Twist2*$^{f/f}$ mice.

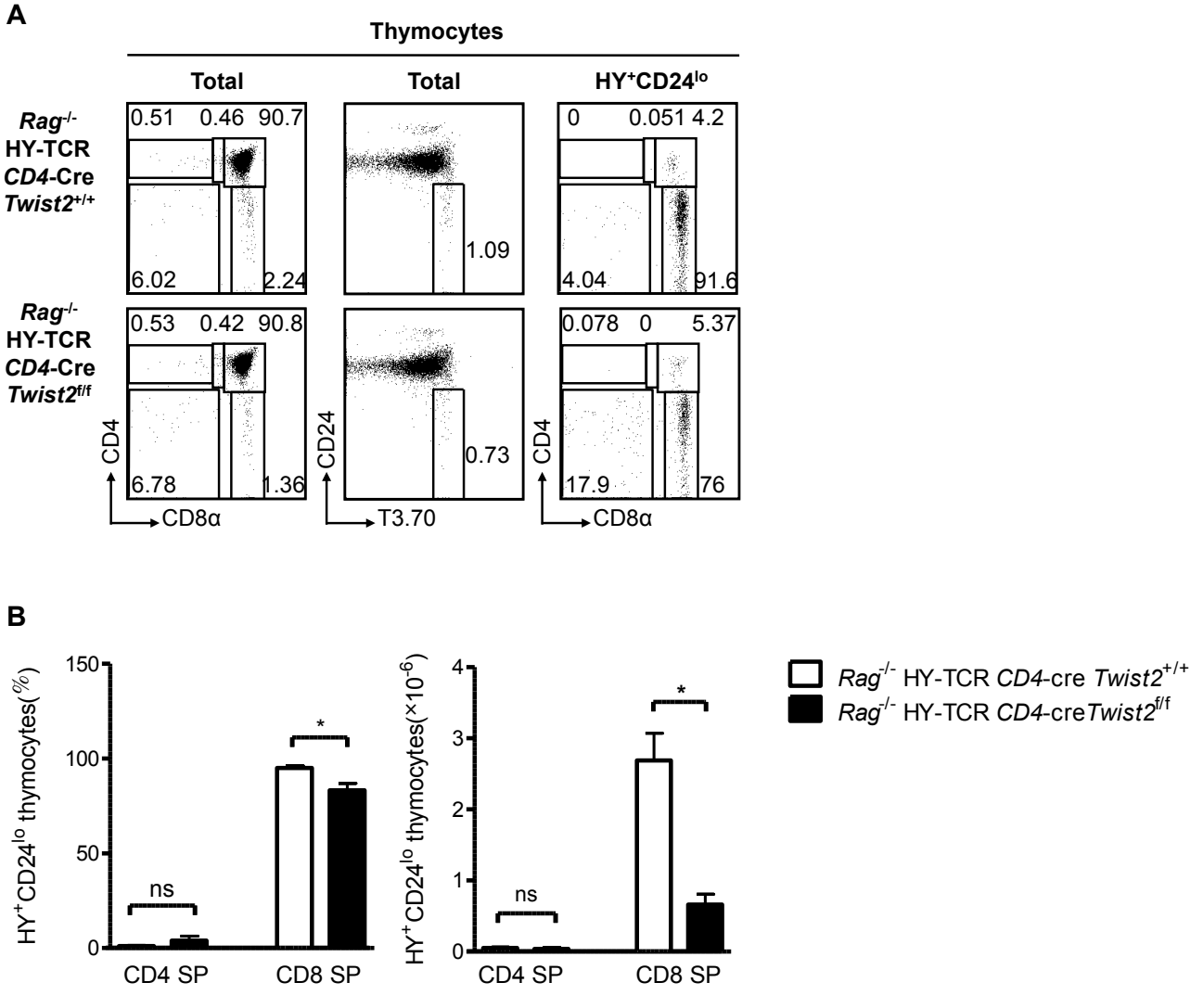
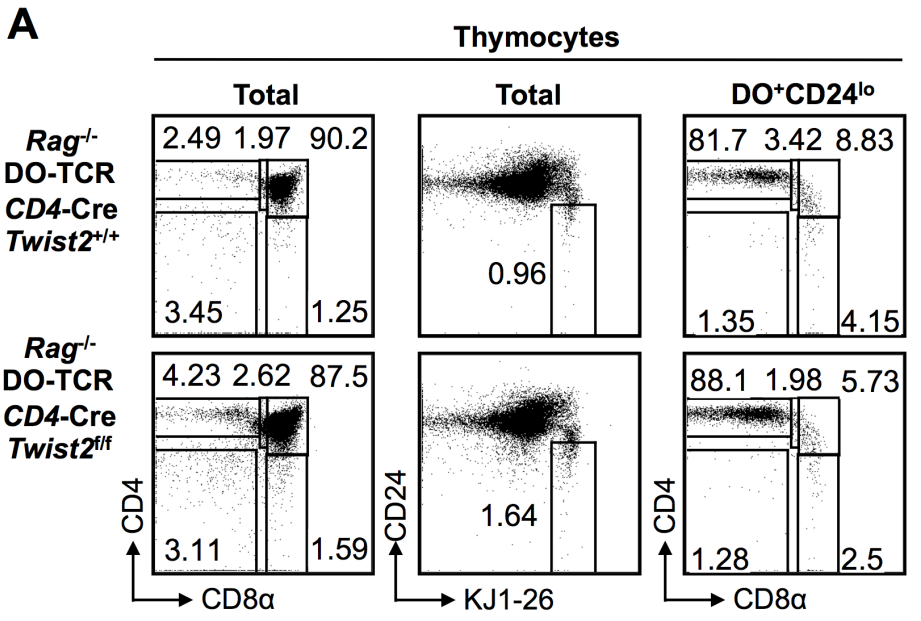
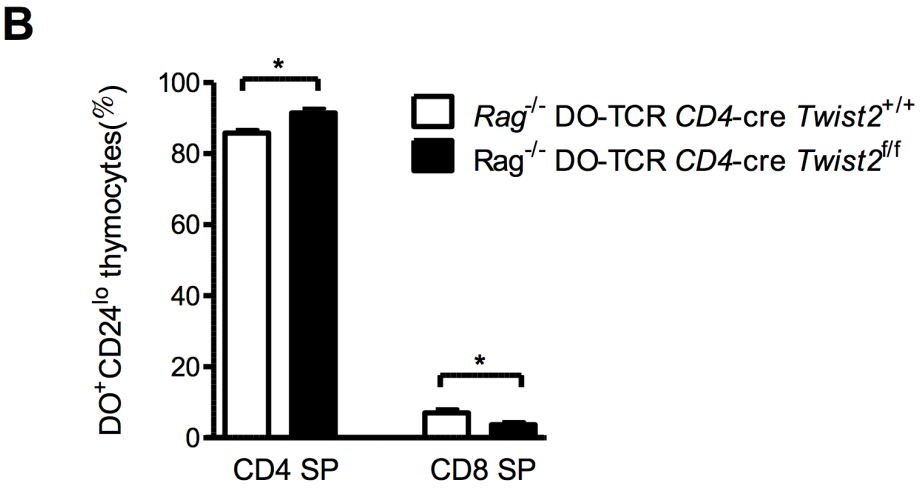

A
Thymocytes
Total
Total
HY+CD24lo
Rag-/- HY-TCR CD4-Cre Twist2+/+
0.51 0.46 90.7
6.02 2.24
1.09
0 0.051 4.2
4.04 91.6
Rag-/- HY-TCR CD4-Cre Twist2f/f
0.53 0.42 90.8
6.78 1.36
0.73
0.078 0 5.37
17.9 76
CD4
CD8α
CD24
T3.70
B
HY+CD24lo thymocytes(%)
150
100
50
0
ns
*
CD4 SP
CD8 SP
HY+CD24lo thymocytes(×10-6)
4
3
2
1
0
ns
*
CD4 SP
CD8 SP
Rag-/- HY-TCR CD4-cre Twist2+/+
Rag-/- HY-TCR CD4-creTwist2f/f

Figure 28. CD4 SP thymocytes were slightly increased in *Twist2* cKO mice with MHC class II-restricted TCR-transgenic background.

(A) CD4 and CD8α expression in total thymocytes and $DO^{+}CD24^{lo}$ thymocytes from *Rag*$^{-/-}$ DO-TCR *CD4*-Cre *Twist2*$^{+/+}$ mice and *Rag*$^{-/-}$ DO-TCR *CD4*-Cre *Twist2*$^{f/f}$ mice. (B) Frequency of mature CD4 SP and CD8 SP thymocytes in (A).


A
Thymocytes
Total
Total
DO+CD24lo
Rag-/-
DO-TCR
CD4-Cre
Twist2+/+
2.49 1.97 90.2
3.45
1.25
0.96
81.7 3.42 8.83
1.35
4.15
Rag-/-
DO-TCR
CD4-Cre
Twist2f/f
4.23 2.62 87.5
3.11
1.59
1.64
88.1 1.98 5.73
1.28
2.5
CD4
CD8α
CD24
KJ1-26
CD4
CD8α


B
DO+CD24lo thymocytes(%)
100
80
60
40
20
0
*
*
Rag-/- DO-TCR CD4-cre Twist2+/+
Rag-/- DO-TCR CD4-cre Twist2f/f
CD4 SP
CD8 SP

mice, *B2m*$^{-/-}$ *Twist2* transgenic mice generated significant numbers of CD8 SP cells in the thymus (**Figure 29**). Concurrently, the percentage of TCRβ^{+} CD4 SP thymocytes was significantly decreased in *B2m*$^{-/-}$ *Twist2* transgenic mice. In the periphery, *B2m*$^{-/-}$ *Twist2* transgenic mice displayed significant numbers of CD8 SP splenocytes.

I also analyzed the effect of Twist2 deficiency on CD4/CD8 lineage differentiation under MHC class II-deficient background. The overall thymic cellularity was decreased in *MHC class II*$^{-/-}$ *CD4*-Cre *Twist2*$^{f/f}$ mice compared with *MHC class II*$^{-/-}$ *CD4*-Cre *Twist2*$^{+/+}$ mice, and there was a significant reduction in the numbers of TCRβ^{+}CD24^{lo} CD8 SP thymocytes in *Twist2* cKO mice (**Figure 30**). It is also clear that CD8 SP differentiation is severely affected when splenocytes were analyzed. However, there was only minor, and no significant, change in CD4 SP cells in MHC class I-restricted thymocytes from *Twist2* cKO mice.

In addition, I carried out bone marrow transplantation of *Twist2* cKO mice into *MHC class II*$^{-/-}$ mice. Lethally irradiated (1,100 rad) *MHC class II*$^{-/-}$ recipients were reconstituted with 1:1 mixture of T-cell depleted bone marrow from congenic CD45.1^{+} mice and from either CD45.2^{+} *lck*-Cre *Twist2*$^{+/+}$ or *lck*-Cre *Twist2*$^{f/f}$ mice. Similarly to *MHC class II*$^{-/-}$ *CD4*-Cre *Twist2*$^{f/f}$ mice, only minor, and no significant, increase in CD4 SP thymocytes was observed (**Figure 31**).

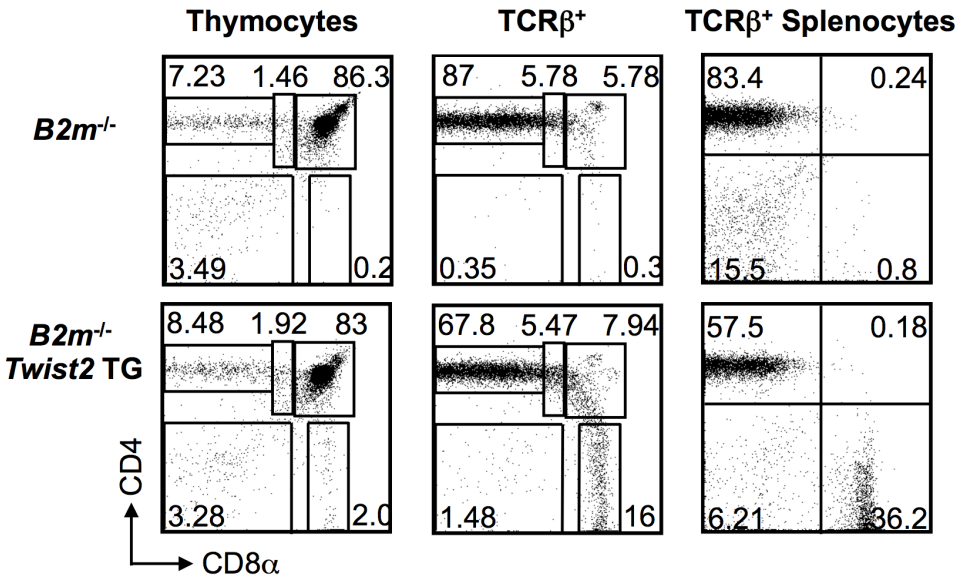
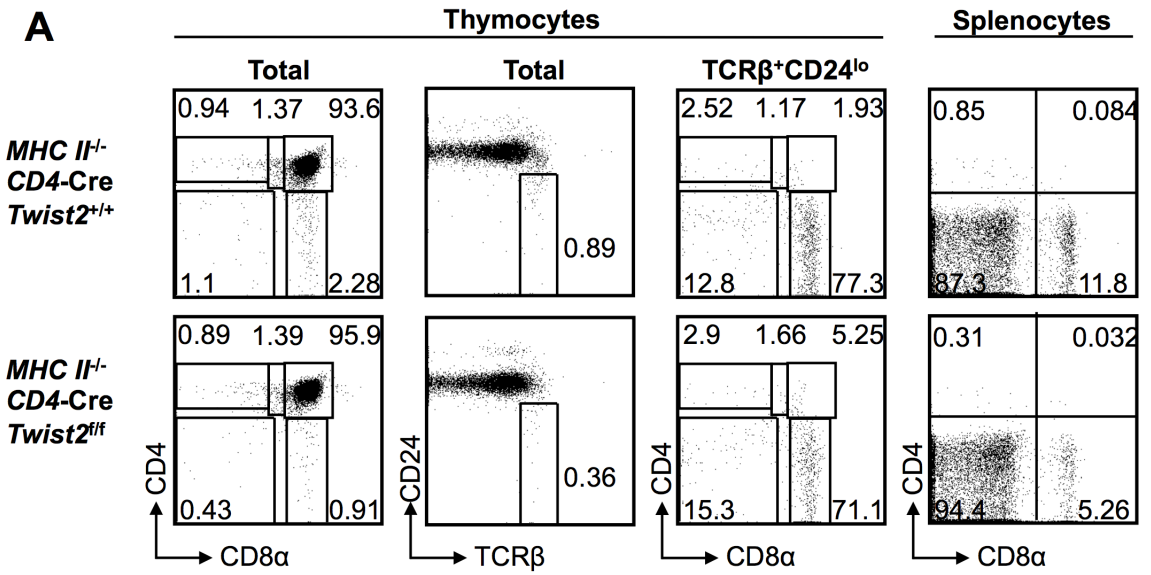


Figure 29. *B2m*$^{-/-}$ *Twist2* transgenic mice generated significant numbers of CD8 SP cells in the thymus.

Thymocyte differentiation profiles based on CD4 and CD8α expression and TCRβ expression of total thymocytes, and CD4 and CD8α expression of TCRβ^{+} thymocytes and splenocytes from the indicated mice.

Figure 30. The numbers of TCRβ^{+}CD24^{lo} CD8 SP thymocytes was reduced in *MHC class II*$^{-/-}$ *CD4*-Cre *Twist2*$^{f/f}$ mice compared with *MHC class II*$^{-/-}$ *CD4*-Cre *Twist2*$^{+/+}$ mice.

(A) Thymocyte differentiation profiles based on the indicated surface markers from the *MHC class II*$^{-/-}$ *CD4*-Cre *Twist2*$^{f/f}$ mice and control mice. (B) Cell numbers of CD8 SP from the indicated mice.


A
Thymocytes
Splenocytes
Total
Total
TCRβ+CD24lo
MHC II-/- CD4-Cre Twist2+/+
MHC II-/- CD4-Cre Twist2f/f
0.94 1.37 93.6
1.1 2.28
0.89
2.52 1.17 1.93
12.8 77.3
0.85 0.084
87.3 11.8
0.89 1.39 95.9
0.43 0.91
0.36
2.9 1.66 5.25
15.3 71.1
0.31 0.032
94.4 5.26
CD4
CD8α
CD24
TCRβ

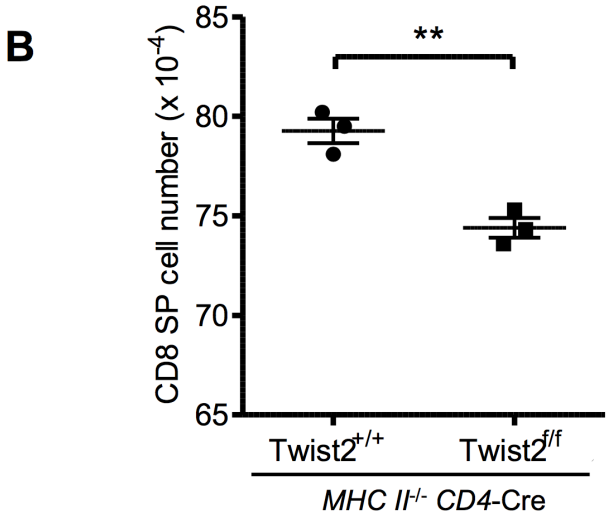
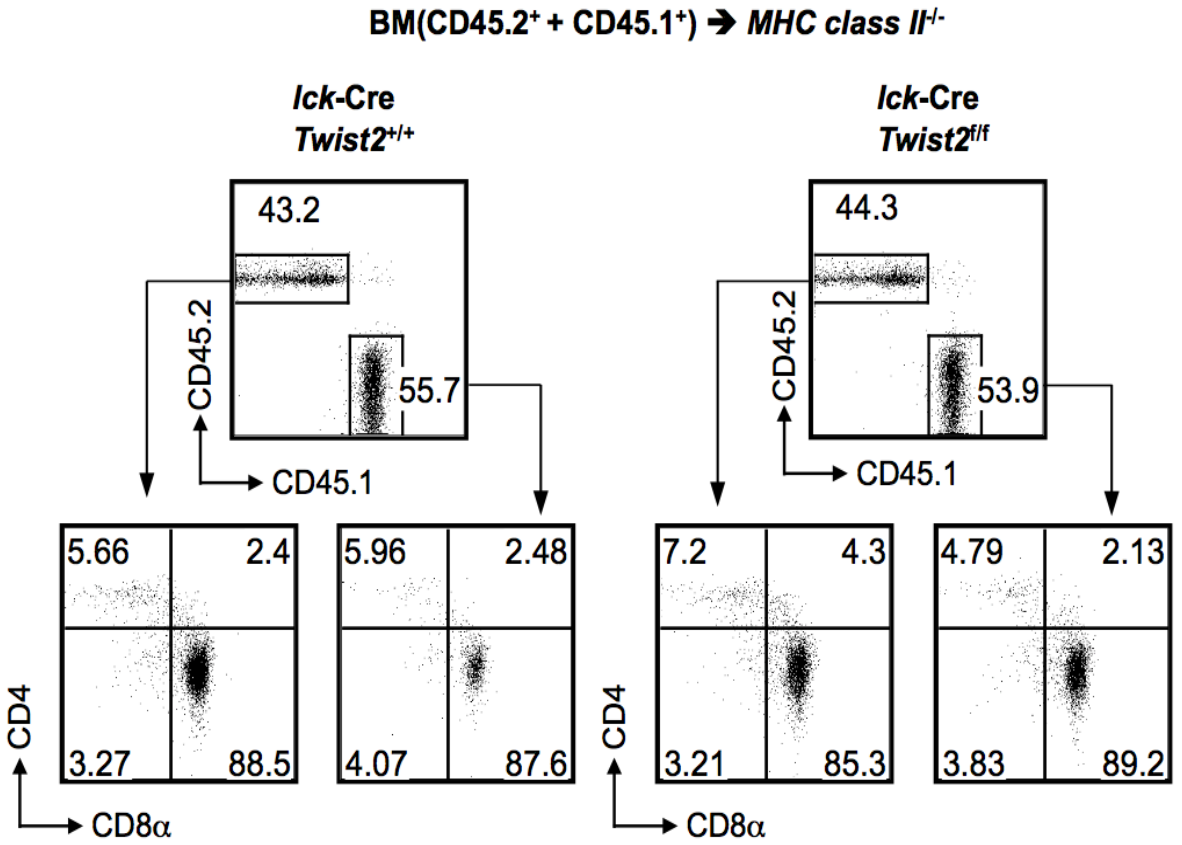

B
CD8 SP cell number (x 10-4)
85
80
75
70
65
**
Twist2+/+
Twist2f/f
MHC II-/- CD4-Cre

Figure 31. Bone marrow transplantation of Twist2 cKO mice into MHC class II-/- mice.

CD45.1 and CD45.2 expression (above) on thymocytes from irradiated *MHC class II*$^{-/-}$ recipients of *lck*-Cre *Twist2*$^{+/+}$ (left) or *lck*-Cre *Twist2*$^{f/f}$ (right) CD45.2^{+} bone marrow mixed at a ratio of 1:1 with wild-type (CD45.1^{+}) bone marrow. TCRβ^{+}CD24^{lo} CD4/CD8 SP cells from each CD45.1^{+} and CD45.2^{+} thymocytes were presented (below).


BM(CD45.2+ + CD45.1+) ➔ MHC class II-/-
lck-Cre
Twist2+/+
lck-Cre
Twist2f/f
43.2
55.7
44.3
53.9
CD45.2
CD45.1
5.66
2.4
3.27
88.5
5.96
2.48
4.07
87.6
7.2
4.3
3.21
85.3
4.79
2.13
3.83
89.2
CD4
CD8α

To test how efficiently CD4 SP thymocytes generated compared with *MHC class II*$^{-/-}$ *CD4*-Cre *Twist2*$^{f/f}$, I analyzed *Runx3*-, *Mazr*-, or *CBFb*-conditional knock out mice with MHC II $^{-/-}$ background. CD4/CD8 profile is similar between *MHC class II*$^{-/-}$ *CD4*-Cre *Mazr*$^{f/f}$ and *MHC class II*$^{-/-}$ *CD4*-Cre *Twist2*$^{f/f}$ (**Figure 32**). As reported, *Runx3*- and *CBFb*-conditional knock out mice showed CD4 derepression, so mature DP (TCRb^{high},CD24^{lo}, CD4^{+}CD8^{+}) cells appeared. Also, *CBFb*-conditional knock out mice showed significant increase of CD4^{+} thymocytes. To confirm whether generated CD4^{+} cells and mature DP cells are functionally CD4 SP or not, these cells were sorted and checked for the *ThPOK* and *perforin* mRNA level. As a result, *ThPOK* expression level is low and *perforin* level is high in both CD4^{+} cells and mature DP cells (**Figure 33**). Therefore, CD4^{+} cells in *Runx*- or *CBFb*-conditional knock out mice are merely phenotypical CD4^{+} that are not functional CD4 SP cells.

As it was reported that Twist1 and Twist2 exert similar functions in various tissues, I investigated the possible redundancy of Twist proteins in CD4/CD8 lineage differentiation. In contrast to *Twist2* cKO mice, *Twist1* cKO mice showed no significant changes in thymocyte differentiation profile. There was no significant effect on ThPOK expression, and no interaction with Runx complex (**Figure 34**). *Twist1* and *Twist2* double cKO mice showed similar results to those obtained from *Twist2* cKO mice (data not shown).

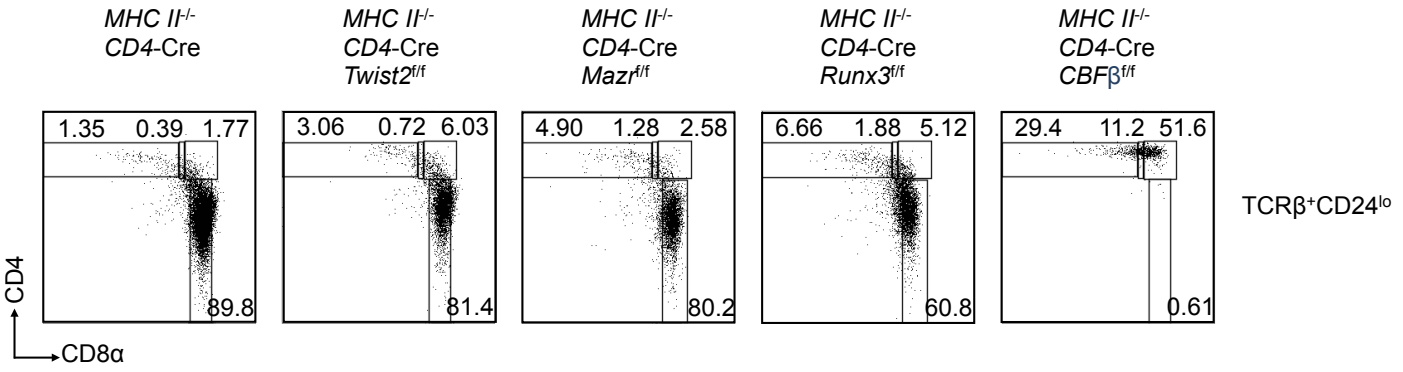


Figure 32. Analysis of *Twist2*-, *Mazr* -, *Runx3*-, and *CBFb*-deficient mice with *MHC class II*$^{-/-}$ background.

Thymocyte differentiation profiles based on the indicated surface markers from indicated mice.

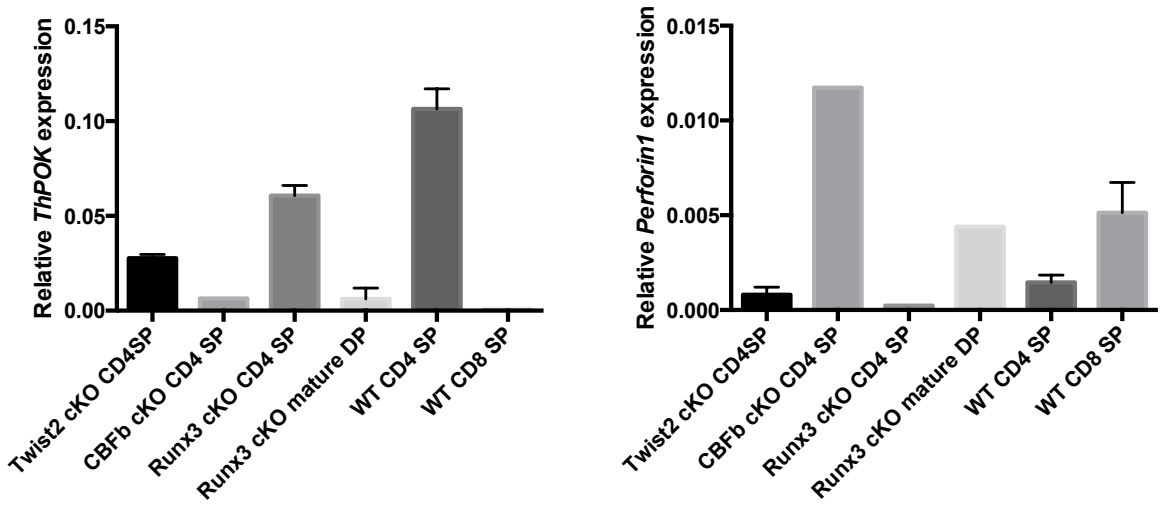
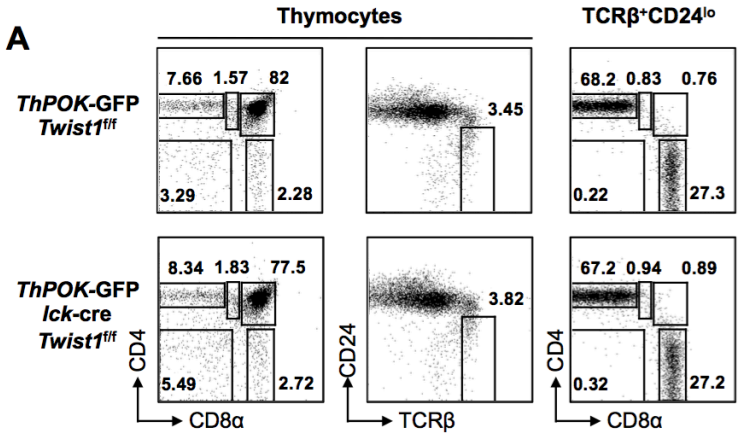


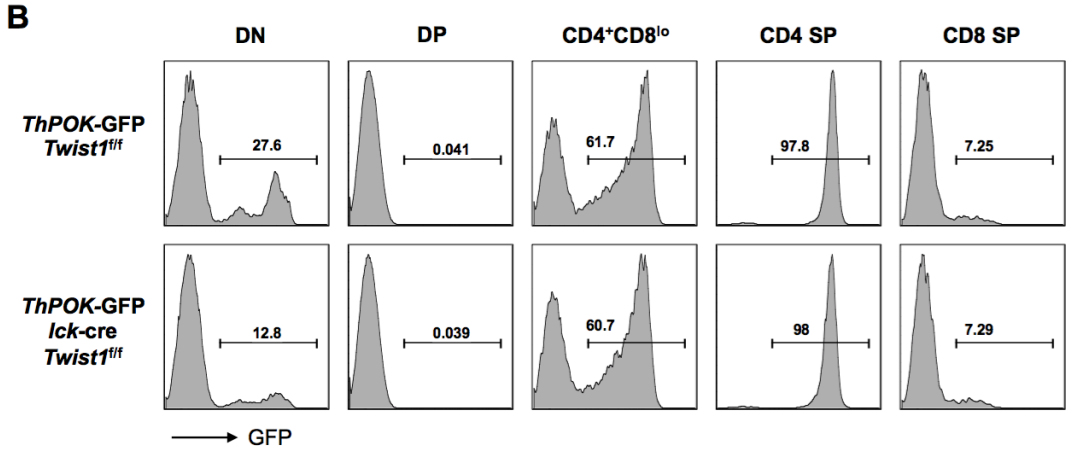
Figure 33. CD4$^+$ cells in *Runx*-conditional knock out mice are merely phenotypical CD4$^+$ that are not functional CD4SP cells.

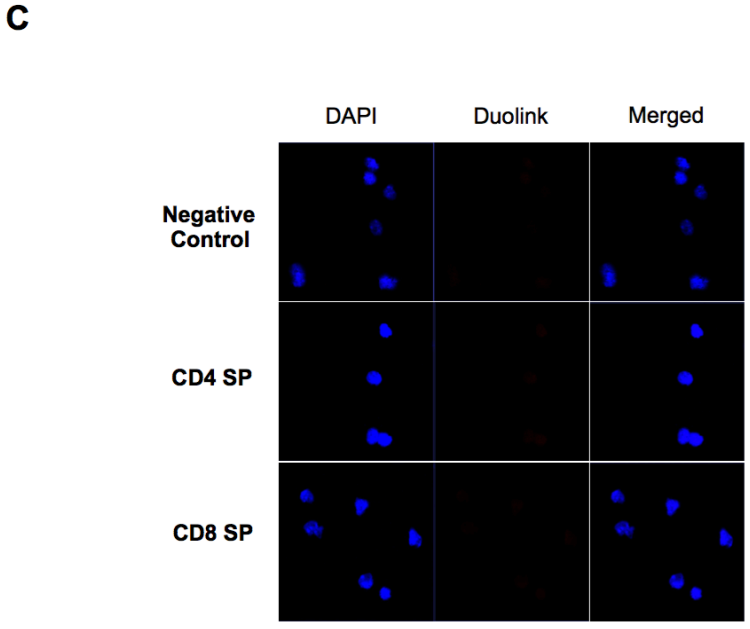
ThPOK and *Perforin1* expression was determined by Q-PCR in CD4+ thymocytes and mature DP (TCRb^{high}CD24^{lo}CD4$^+$CD8$^+$) thymocytes from indicated mice sorted by flow cytometry.

Figure 34. Thymocyte-specific Twist1 deficiency is not associated with CD4/CD8 lineage differentiation.

(A) Thymocyte differentiation profiles based on the indicated surface markers from the indicated mice. (B) ThPOK expression was determined by the relative fluorescence intensity of GFP from the indicated thymocyte subsets from the indicated mice. (C) For Duolink *in situ* PLA analysis, the indicated subsets of WT thymocytes were incubated with anti-Twist1 and anti-Cbfβ antibodies, followed by the addition of PLA probes. The fluorescence signal was measured by confocal microscopy.


A
Thymocytes
TCRβ+CD24lo
ThPOK-GFP Twist1f/f
ThPOK-GFP lck-cre Twist1f/f
7.66 1.57 82
3.29 2.28
3.45
68.2 0.83 0.76
0.22 27.3
8.34 1.83 77.5
5.49 2.72
3.82
67.2 0.94 0.89
0.32 27.2
CD4
CD8α
CD24
TCRβ


B
DN
DP
CD4+CD8lo
CD4 SP
CD8 SP
ThPOK-GFP Twist1f/f
ThPOK-GFP lck-cre Twist1f/f
27.6
0.041
61.7
97.8
7.25
12.8
0.039
60.7
98
7.29
GFP


C
DAPI
Duolink
Merged
Negative Control
CD4 SP
CD8 SP

DISCUSSION

CD4/CD8 T cell lineage commitment is key issues in the development of the immune system. Several transcription factors are known to participate during CD4/CD8 lineage commitment. Particularly, ThPOK seems to be both necessary and sufficient for CD4-lineage differentiation and Runx3 plays an important role in CD8 SP differentiation by repressing ThPOK expression. However, Runx complex binds persistently to the silencer region of *ThPOK* in thymocyte subsets, regardless of their capability to differentiate into the CD4 or CD8 SP lineage. Thus, some other factor would act to resolve the distinct suppressive activity of the Runx complex on *ThPOK* between CD4 and CD8 SP cells. Here, I showed that Twist2 expression is finely regulated during thymocyte development and differentiation, and it plays a critical role during CD4/CD8 lineage differentiation by controlling *ThPOK* expression through its interaction with the Runx complex.

Twist2 expression is differentially regulated by T-cell receptor signaling leading to differentiation into the CD4 or CD8 lineage

Transcription factor Twist2 is tightly regulated during thymocyte development in a differentiation stage-specific manner. Twist2 is upregulated at the $CD4^{+}CD8^{lo}$ stage when thymocytes undergo positive selection through TCR-MHC engagement. Interestingly, Twist2 upregulation was negatively

correlated with the intensity of the activation signal, i.e., a weak activation signal for CD8 SP differentiation induced much higher activation of *Twist2* expression than a strong signal for CD4 SP differentiation (**Figure 35**). Consistently, Twist2 expression was differentially regulated when thymocytes differentiated into CD4 or CD8 SP cells. Twist2 expression was higher in CD8 SP thymocytes than in CD4 SP thymocytes. These results suggest that Twist2 is directly regulated by TCR signaling, which is crucial for thymocyte selection and lineage differentiation processes.

Twist2 is involved in CD4/CD8 thymocyte lineage determination by regulating a crucial transcription factor, ThPOK, through its interaction with another important factor, the Runx complex.

Recent studies have revealed that various transcription factors are involved in CD4/CD8 lineage commitment(He et al., 2005; Hernandez-Hoyos et al., 2003; Pai et al., 2003; Sakaguchi et al., 2010; Setoguchi et al., 2008; Sun et al., 2005a, b; Wilkinson et al., 2002; Woolf et al., 2003). Among them, ThPOK is a crucial factor for CD4 SP differentiation, and its expression is mainly regulated by Runx complexes. However, persistent binding of Runx complexes to the silencer region of the *ThPOK* promoter, regardless of the differentiation stage, raised the possible involvement of other factor(s) in the regulation of ThPOK expression. Twist2 was previously shown to interact with Runx1 and Runx2, and its interaction with Runx3 was also reported recently(Pham et al., 2012; Sharabi et al., 2008; Yang et al., 2011). I demonstrated that Twist2

interacts with Runx3 by Co-IP and Duolink assay, and that the interaction between Twist2 and the Runx complex is stage-specific by using the Duolink assay. Furthermore, a ChIP assay suggested that Twist2 could bind to the silencer region of the *ThPOK* promoter in a lineage-dependent manner. Biotin pull-down assay using biotinylated oligomers corresponding to NF-κB / E box region and Runx binding region of ThPOK silencer showed that Twist2 was specifically bound to the Runx binding sites. It appears that there is a difference between the interaction of Twist2 with Runx during CD4 and CD8 lineage differentiation (**Figure 35**). Previous reports have shown that Twist2 interacts with Runx proteins, and thereby inhibits their transcriptional activity(Sharabi et al., 2008; Yang et al., 2011). In addition, Twist2 expression was associated with the transcriptional downregulation of *Runx* genes(Sharabi et al., 2008). However, Twist2 did not suppress the expression of *Runx3* (data not shown) in this system. Moreover, like Runx3, Twist2 downregulated the expression of *ThPOK in vitro* and *in vivo* (**Figure 6** and **Figure 13**), and a ChIP assay showed that Twist2 binds only to the silencer region among the two major Runx targets. These results suggest that the Twist2 and Runx complex interaction in this system resulted in the cooperative suppression of *ThPOK*. This kind of cooperation between Twist2 and Runx3 was also suggested by results obtained from the *Twist2* and *Runx3* double transgenic mice, in which CD4 expression was much more severely affected (**Figure 18**). This cooperative regulation by Twist2 and Runx3 may not be limited to *ThPOK* but may be extended to *Cd4* and other targets on which Runx3 acts as a repressor.

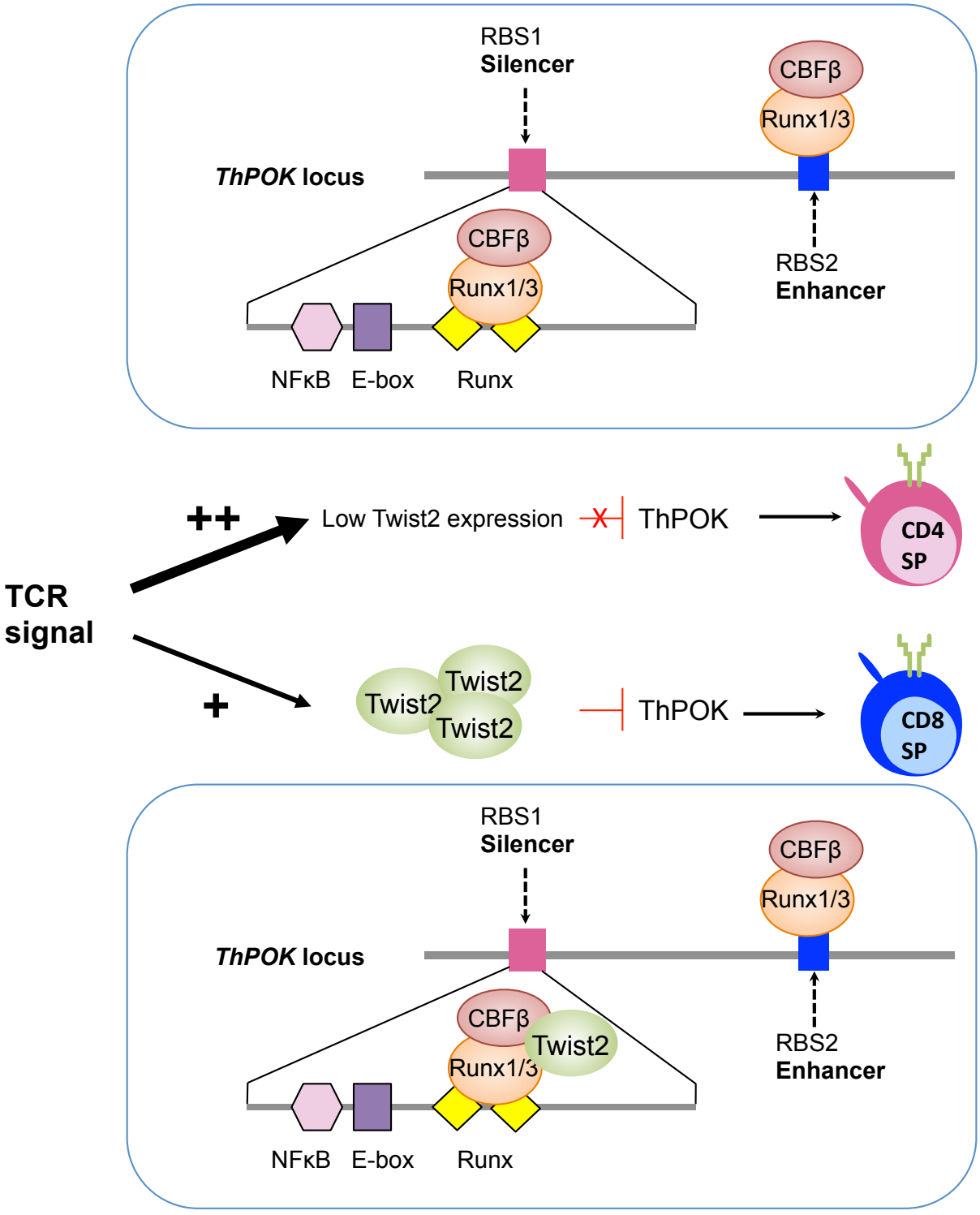


Figure 35. A schematic diagram illustrating a role for Twist2 in CD4/CD8 lineage commitment.

Twist2 was associated with altered CD4/CD8 thymocyte lineage differentiation

The ratio of TCRβ^{+}CD24^{lo} CD4/CD8 SP thymocytes was significantly decreased in *Twist2* transgenic mice, whereas the ratio was significantly increased in *Twist2* cKO mice (**Figures 13C** and **21C**). In MHC class II-restricted TCR transgenic mice, Twist2 impaired TCR-transgenic CD4 SP development, whereas significant numbers of MHC class II-restricted CD8 SP thymocytes and splenocytes were generated (**Figure 24**). In contrast, the development of CD8 SP thymocytes expressing MHC class I-restricted TCR was significantly impaired by Twist2 deficiency (**Figure 26**). Further investigation with *B2m*$^{-/-}$ mice demonstrated that Twist2 could generate MHC class II-restricted CD8 SP cells (**Figure 29**). As I can rule out any effects of endogenous TCRs in TCR transgenic mice, this result can be interpreted as lineage conversion. The lineage conversion was closely related with the differential binding of Twist2 to the Runx complex and RBS1 region of *ThPOK* in CD4^{+}CD8^{lo} thymocytes, resulting in the distinct expression of ThPOK in these thymocytes.

The enforced expression of Twist2 converts MHC class II-restricted cells to CD8 SP cells by direct repression of ThPOK expression, but unlikely by CD4 repression

It has been reported previously that Runx3 binds to the silencer regions of both *CD4* and *ThPOK* and suppresses the expression of those genes and Runx3 transgenic mice exhibit substantial redirection of MHC class II-restricted thymocytes into the CD8 lineage, but this effect can be corrected with a CD4 transgene, indicating that it is an indirect consequence of CD4 downmodulation (or additional effects on ThPOK expression). Because if CD4 expression is suppressed in DP thymocytes, chance of MHC class II-CD4 interaction is decreased, leading to perturbed CD4 SP differentiation. To check the possibility that the aberrant CD4/CD8 lineage differentiation in *Twist2* transgenic mice may result from the downregulation of CD4 as observed in *Runx3* transgenic mice. Twist2 is likely to suppress CD4 expression in DP thymocytes. This was observed in a line of *Twist2* transgenic mice (provided in the Figures). After I observed decreased CD4 expression in Twist2-transfected thymoma cell lines and *Twist2* transgenic mice, I tested whether Twist2 can bind CD4 cis-elements, including promoter, proximal enhancer, silencer and distal enhancer using ChIP assay. The results suggested that Twist2 may be associated with proximal enhancer and silencer regions (data not shown). However, I focus more on Twist2-mediated ThPOK repression because

1) CD4 downregulation was modest in *Twist2* transgenic mice and another line of *Twist2* transgenic mice showed a similar CD4/CD8 SP differentiation pattern with no significant change in CD4 expression

2) ThPOK is regulated by Twist2 and supported by results that ThPOK was downregulated in Twist2-transfected thymoma cell lines regardless of TCR stimuli and *Twist2* conditional knock out results.

3) Furthermore, I investigated the thymocyte differentiation profile of *Twist2* and *CD4* double transgenic mice. The electronically gated $TCR\beta^{hi}CD24^{lo}$ cells from *Twist2* and *CD4* double transgenic mice showed a similar decrease in CD4/CD8 SP ratio to those in *Twist2* transgenic mice (**Figure 19**).

4) Also, I have analyzed OT2-TCR, *Twist2* and *CD4* Triple transgenic mice whether Twist2 can affect MHC class II-restricted cells with constitutive *CD4* transgene. OT2-TCR transgenic mice had few $CD8^+$ cells (<3%) but OT2-TCR and *CD4* double transgenic mice had far increased mature $CD4^+CD8^+$ cells (i.e. CD8 SP) (25~35%). These results are quite similar to the ones reported in ref. (Davis et al., 1993), where mature $CD4^+CD8^+$ cells with cytotoxic function were observed in $B2m^{-/-}$ or PCC TCR Tg mice with CD4 overexpression, OT2-TCR and *CD4* double transgenic mice also showed mature $CD4^+CD8^+$ cells. Analysis of OT2-TCR, *CD4* and *Twist2* triple transgenic mice confirmed that mature $CD4^+CD8^+$ T cells increased greatly compared to the controls (**Figure 20**).

These results support that the enforced expression of Twist2 converts MHC class II-restricted cells to CD8 SP cells by direct repression of ThPOK expression, rather than by CD4 repression. Therefore, I concluded that the Twist2 transgenic mice phenotype is not a secondary effect of CD4 repression but a consequence of Twist2 redirecting CD4 T cells into the CD8 lineage.

Possible reasons why Twist2 deficiency could not efficiently generate MHC class I-restricted CD4 SP cells

As for the loss-of-function of Twist2, the CD4/CD8 SP ratio was increased in *Twist2* cKO mice. The increased CD4/CD8 SP ratio can be caused by increase in CD4 SP cells, decrease in CD8 SP cells, or both. When slightly lower thymic cellularity and decreased $TCR\beta^{+}CD24^{lo}$ population in *Twist2* cKO mice are taken into consideration, It is apparent that the cell numbers of CD8 SP cells are much more severely decreased ($p<0.01$), while the decrease in CD4 SP cell numbers is not statistically significant (**Figures 21B** and **21D***)*. Similar results could be obtained in *Rag*$^{-/-}$ HY-TCR transgenic *Twist2* cKO mice, and *MHC II*$^{-/-}$ *Twist2* cKO mice (**Figures 26B** and **30B**). These results raised the possible involvement of Twist2 in lineage conversion, but Twist2 deficiency could not efficiently generate MHC class I-restricted CD4 SP cells under MHC class II-deficient background (**Figure 30**). I think there are three possibilities to explain this inefficient redirection.

Since Twist2 acts as a repressor, ThPOK repression by Twist2 is more apparent in *Twist2* transgenic mice. However, in *Twist2* cKO mice, this repression appears to be released, increasing ThPOK expression, though to a modest degree. It was reported that there is a threshold of ThPOK expression that is required for complete commitment to the $CD4^{+}$ lineage(Muroi et al., 2008). I think the presence of repressors combined with the near absence of activators leads to low levels of ThPOK expression in $CD4^{+}CD8^{lo}$ thymocytes of CD8 differentiation condition (*MHC II* KO or CD8 SP derived TCR Tg), so

ThPOK expression did not reach the threshold. Since thymocytes of MHC II knock out mice are cells signaled to become CD8 SP, a factor required to induce ThPOK may not be present in these cells, resulting in only modest induction of ThPOK due to absence of Twist2 and thus poor conversion into CD4 lineage cells.

I would like to suggest another explanation to the minor redirection in *MHC II* and *Twist2* double knock out mice. ThPOK silencer has two important regions, which are an essential 80bp core sequence (containing NFkb and E box) and a Runx binding domain. He et al. (2008) showed that ThPOK was not fully derepressed with only E box mutation or only Runx binding domain deletion. Only DRE (or RBS1) deletion or simultaneous deletion of both E box and Runx binding domain induced ThPOK derepression. Therefore, I think both sites (E box domain and Runx binding domain) are necessary for silencer activity. Runx3 binds to Runx binding domain in the RBS1 region, after which Twist2 interacts with Runx3, while unknown proteins bind to NFkB and E box region of RBS1. This combination can suppress the ThPOK expression, so deletion of each protein did not show a dramatic effect because suppression at the other site remains intact. Therefore, I think that derepression effect will become clear when protein interaction at both sites are disrupted.

Lastly, it is possible that epigenetic modification may have already occurred in $CD4^{+}CD8^{lo}$ cells of *MHC II*$^{-/-}$ mice. Tanaka et al. (2013) checked modification of histone H3 lysine residues of ThPOK locus, especially H3K4me3 and H3K27me3 that mark active and repressive states, respectively(Tanaka et al., 2013). In CD4 SP, active H3K4me3 deposition was

detected in silencer, promoter, and proximal enhancer regions but repressive H3K27me3 was almost not detected. On the other hand, in CD8 SP, repressive H3K27me3 was highly detected in silencer region but H3K4me3 signal was not detected. Muroi et al. (2008) showed ThPOK expression patterns in $CD4^{+}CD8^{lo}$ cells in several mice. ThPOK expression was increased about two-fold in *B2m*$^{-/-}$ mice and decreased to about 1/10 in *MHC II*$^{-/-}$ mice compared with WT mice. Also, although Runx complex and MAZR are well known regulators of ThPOK expression and CD4/CD8 lineage commitment, MHC class-I restricted CD4 SP cells are actually not efficiently generated in *Runx1/Runx3* double cKO mice and *MAZR* KO mice, as in *Twist2* cKO mice (**Figures 32** and **33**). These results imply that epigenetic modification (low active marks and high repressive marks) of silencer region has already occurred in $CD4^{+}CD8^{lo}$ in MHC $II^{-/-}$ mice, so repressor proteins do not need to bind and suppress the ThPOK expression. Also, proximal enhancer may have low amount of active H3K4me3 in $CD4^{+}CD8^{lo}$ in *MHC II*$^{-/-}$ mice, so it is hard to fully increase the ThPOK expression. Therefore, I think that ThPOK expression increase modest and MHC class-I restricted CD4 SP is not efficiently generated in MHC $II^{-/-}$ background mice.

Twist1 deficiency is not associated with CD4/ CD8 lineage differentiation

Twist1 and Twist2 proteins share high structural homology. Both contain a basic helix-loop-helix DNA binding domain, and can form homodimers or

heterodimers with another bHLH protein through this domain(Li et al., 1995; Sosic et al., 2003). Moreover, Twist proteins share the Twist box in the C-terminus, which is important for interaction with other proteins including Runx proteins. In an attempt to investigate the redundant roles of Twist proteins during CD4/CD8 lineage differentiation, I analyzed *Twist1* cKO and *Twist1* and *Twist2* double cKO mice. I found no evident role of Twist1 during the differentiation in view of thymocyte differentiation profile, ThPOK expression, and interaction with Runx complex (**Figure 34**). Based on these findings, it appears that there is no functional redundancy between Twist1 and Twist2 at least during the CD4/CD8 thymocyte lineage differentiation.

Potential additional role of Twist2 that interacts with transcriptional factors that are significantly implicated in CD4/CD8 lineage differentiation

In view of the transcription factor network that governs the CD4/CD8 lineage differentiation of DP thymocytes, Twist2 may serve as an early player during lineage specification based on its TCR signaling-dependent regulation. Twist2 may regulate the expression of ThPOK also through the regulation of other upstream factors in addition to its direct activity on the silencer region of the *ThPOK* promoter. ThPOK expression was shown to be dependent on Gata3, and Gata3 is regulated by c-Myb(Maurice et al., 2007; Wang et al., 2008). Although I found that Twist2 could downregulate *Gata3* to some extent *in vitro* (**Figure 6**), the precise regulatory mechanisms remain to be identified. Recently, it was reported that E proteins are required for the development of

CD4 lineage T cells; persistent E protein activity during positive selection may permit CD4 lineage development while preventing CD8 lineage development(Jones-Mason et al., 2012). Twist2 was shown to form a heterodimer with E proteins and also interact with the NF-κB transcription factor(Sosic et al., 2003). Although Twist2 did not bind to NK-κB or the E box fragment of the silencer region of *ThPOK* (**Figure 8A**), it is possible that Twist2 is involved in the E-protein-dependent regulation of *Gata3*.

In summary, my results demonstrate that *Twist2* is activated by TCR signaling, and its lineage-specific expression pattern and interaction with the Runx complex are involved in the regulation of *ThPOK* expression during CD4/CD8 lineage differentiation. The gain-of-function and loss-of-function mutations in *Twist2* showed its critical role in CD4/CD8 SP lineage differentiation. The identification of Twist2 as a novel key player in CD4/CD8 T cell lineage differentiation provides another important clue to understanding the molecular mechanism underlying the differentiation of thymocytes into CD4/CD8 lineage SP cells. Furthermore, my results clearly offer the answer to the key question of how Runx represses the expression of ThPOK. Runx complex is bound to both the silencer and the enhancer region of ThPOK throughout T cell differentiation. Thus the differential regulation of ThPOK expression in CD4/CD8 lineage differentiation could not be explained by Runx alone. My data provide critical evidence of Twist2 as a new factor that is regulated by TCR signaling strength and in turn directly represses the

expression of ThPOK, and thereby, functions during the CD4/CD8 lineage differentiation.

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국 문 초 록

CD4/CD8 T 세포 계열 결정 과정에서 Twist2 의 기능 연구

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CD4/CD8 T 세포 계열 결정은 면역시스템 발달에 중요하다. TCR 신호의 강도와 지속시간이 CD4/CD8 계열을 결정하는 중요 인자로 알려져 있다. 또한 CD4/CD8 T 세포 계열 결정에 관여하는 여러가지 전사인자들이 밝혀져있다. Gata3 와 ThPOK 은 CD4 T 세포로 분화하는데 중요하다고 알려져 있는 반면, Runx3 와 Mazr 는 CD8 T 세포로 분화하는데 중요한 역할을 한다고 알려져 있다. 특히 ThPOK 단백질의 발현은 CD4 T 세포로 분화하는데 필요 충분조건으로 여겨지고, Runx3 는 ThPOK 발현의 억제를 통해 CD8 T 세포로 분화하는데 역할을 한다는 사실이 잘 알려져 있다. Runx 단백질은 ThPOK 의 silencer 부위에 결합하고 있는데, CD4 T 세포나 CD8 T 세포에 관계없이 항상 결합되어 있음이 알려져있다. 이러한 기존의 연구들은 지금까지 보고되지 않은 새로운 인자가 CD4 T 세포와 CD8 T 세포에서 다르게 ThPOK 의 silencer 에 결합하여 Runx 의 억제 활성을 조절 할 가능성을 제시하고 있다.

Twist2 는 bHLH 전사인자로서 주로 전사 억제자로 작용함이 알려져있다. 최근 Twist2 는 TCR 신호에 의해 그 발현이 조절되며 흉선세포의 생존과

죽음에 관여함이 보고되었다. 특히 Nur77 과 Nor-1 의 발현을 조절하여 'TCR 신호-매개 세포사멸'을 조절한다는 것이 잘 알려져 있다. 이러한 결과들은 Twist2 가 흉선세포 발생과정에 중요한 역할을 할 것이라는 것을 예상해 볼 수 있게 한다.

이 논문은 Twist2 가 CD4/CD8 계열 결정에서 중요하다는 것을 밝히고 있다. Twist2 는 TCR 신호에 의해서 그 발현이 유도되며, 특히 CD4 T 세포와 CD8 T 세포로 가는 TCR 신호에 따라 그 발현 정도가 달라진다. CD8 T 세포로 가는 신호가 왔을 때 더 많이 발현되며, 오래 지속된다. 또한 Twist2 의 발현은 CD4 T 세포에서보다 CD8 T 세포에서 높다. 그리고 Twist2 는 Runx3 와 결합할 수 있고, 이 결합은 Twist2 단백질의 C-말단 부위인 Twist2 box 부분을 통해서 이루어진다. 또한 Twist2 단백질은 Runx3 와 마찬가지로 ThPOK 의 silencer 부위에 결합하여 ThPOK 의 발현을 억제한다. Runx 와의 차이점은 Runx 단백질은 ThPOK 의 silencer 에 항상 붙어있는 반면, Twist2 는 CD8 T 세포 특이적으로 결합한다는 것이다. ThPOK 의 silencer 부위는 두가지 중요부위가 알려져 있는데, NF-kB 와 E box 를 포함하는 필수적인 80bp 의 핵심 서열과 Runx 복합체가 결합할 수 있는 Runx binding domain 이 있다. Twist2 는 특이적으로 Runx bindng domain 에 결합하는 것을 확인하였다. 이 결과들은 Twist2 가 CD8 계열 특이적으로 ThPOK 의 silencer 부위 중 Runx 가 붙어있는 곳에 결합하여 ThPOK 의 발현을 억제한다는 사실은 말해준다. 더욱이 Twist2 과발현 마우스는 CD4 T 세포의 생성이 감소하고, CD8 T 세포의 생성이 증가되어 있다. 또한 CD4 T 세포만 생성되고 CD8 T 세포가 생성되지 않는 $B_2m^{-/-}$ 마우스에서 조차 Twist2 가 과발현된 경우 CD8 T 세포가 생성된 것을 알 수 있다. 반대로 Twist2 결핍마우스의 경우에는 CD8 T 세포로 분화가 유도되는 TCR 마우스에서

조차 CD8 T 세포의 생성이 줄어드는 것을 확인할 수 있다. 이 논문은 유전자 과발현 마우스 시스템과 유전자 결핍 마우스 시스템을 이용하여 Twist2 가 CD8 T 세포 발생 특이적으로 Runx 와의 결합을 통해 ThPOK 의 silencer 부위에 결합하여 ThPOK 의 발현을 억제함으로써 CD4/CD8 계열 결정에 중요하게 작용한다는 사실을 밝혔다.

주요어 : Twist2, CD4/CD8 계열 결정, ThPOK, Runx 복합체

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